TURUN YLIOPISTON JULKAISUJA
ANNALES UNIVERSITATIS TURKUENSIS

SARJA - SER. A I OSA - TOM. 440

ASTRONOMICA - CHEMICA - PHYSICA - MATHEMATICA

FREE PROSTATE-SPECIFIC ANTIGEN FORMS AND KALLIKREIN-RELATED PEPTIDASE 2:

TOOLS FOR PROSTATE CANCER DIAGNOSTICS

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Turku 2012

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ISBN 978-951-29-5051-5 (PRINT)
ISBN 978-951-29-5052-2 (PDF)
ISSN 0082-7002
Painosalama Oy – Turku, Finland 2012

To men

and

to the women in their life

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LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following publications, referred to in the text by the Roman numerals in the following list (**I-IV**):

I Väisänen, V., Peltola, M.T., Nurmi, M., Lilja, H. and Pettersson, K. (2006) Intact Prostate-Specific Antigen (PSA) and Free and Total Human Glandular Kallikrein 2 (hK2) – Elimination of Assay Interference by Enzymatic Digestion of Antibodies to $F(ab')_2$ Fragments. *Anal Chem* **78**(22):7809–7815.

II Peltola, M.T., Niemelä, P., Väisänen, V., Viitanen, T., Alanen, K., Nurmi, M. and Pettersson, K. (2011) Intact and Internally Cleaved Free Prostate-Specific Antigen in Patients With Prostate Cancer With Different Pathologic Stages and Grades. *Urology* **77**:1009.e1–1009.e8.

III Carlsson, S., Peltola, M.T., Sjoberg, D., Schröder, F., Hugosson, J., Pettersson, K., Scardino, P., Vickers, A., Lilja, H. and Roobol, M. (2012) Can one blood draw replace transrectal ultrasound estimated prostate volume? *Submitted to BJUI.*

IV Peltola, M.T., Niemelä, P., Alanen, K., Nurmi, M., Lilja, H. and Pettersson, K. (2011) Immunoassay for the discrimination of free prostate-specific antigen (fPSA) forms with internal cleavages at Lys_{145} or Lys_{146} from fPSA without internal cleavages at Lys_{145} or Lys_{146}. *J Immunol Methods* **369**:74–80.

In addition, some unpublished data is presented.

ABBREVIATIONS

%pPSA	proPSA/fPSA ratio
[-2]proPSA	proPSA form which has a truncated propeptide
A2M	α_2-macroglobulin
ACT	α_1-antichymotrypsin (SERPINA3)
ANN	artificial neural network
API	α_1-antirypsin (SERPINA1)
AT	α_1-antirypsin (SERPINA1)
AUC	area under the curve
BPH	benign prostatic hyperplasia
BPSA	benign prostate-specific antigen
BSA	bovine serum albumin
cPSA	complexed prostate-specific antigen
DRE	digital rectal examination
EDTA	ethylenediaminetetraacetic acid
ek-hK2	enterokinase hK2, mutant recombinant prohK2
ERSPC	The European Randomized study of Screening for Prostate Cancer
Eu	europium
$F(ab')_2$	Ig antigen binding fragment consisting of two Fab fragments bound together by a disulphide bridge
F/T PSA	fPSA / tPSA
Fab	Ig antigen binding fragment consisting of light chain and part of the heavy chain
Fc	fragment crystallisable region of Ig
fhK2	free kallikrein-related peptidase 2
fPSA	free prostate-specific antigen
fPSA(2C1)	immunoassay for measuring fPSA, Mab 2C1 as the tracer
fPSA-I	intact free prostate-specific antigen
fPSA-N	nicked free prostate-specific antigen
HAAA	human anti-animal antibody
HAMA	human anti-mouse antibody
HGPIN	high-grade prostatic intraepithelial neoplasia
hK2	kallikrein-related peptidase 2, KLK2
I/F PSA	fPSA-I / fPSA
I/T PSA	fPSA-I / tPSA
Ig	immunoglobulin
IgG	immunoglobulin G
IgM	immunoglobulin M
KLK2	kallikrein-related peptidase 2, hK2
KLK3	kallikrein-related peptidase 3, PSA
KLK4	kallikrein-related peptidase 4
LGPIN	low-grade prostatic intraepithelial neoplasia
Mab	monoclonal antibody
N/T PSA	fPSA-N / tPSA
p2PSA	immunoassay for measuring [-2]proPSA

PCI	protein C inhibitor (SERPINA5)
PCPT	the Prostate Cancer Prevention Trial
phi	prostate health index by Beckman Coulter
PIN	prostatic intraepithelial neoplasia
PLCO	The North American Prostate, Lung, Colorectal, and Ovarian Cancer Screening Trial
prohK2	pro-form (zymogen) of kallikrein-related peptidase 2
proPSA	pro-form (zymogen) of prostate-specific antigen
PSA	prostate-specific antigen, kallikrein-related peptidase 3, KLK3
PSA-ACT	complex of prostate-specific antigen and α_1-antichymotrypsin (SERPINA3)
PSAD	total prostate-specific antigen concentration divided by prostate volume
PSAV	the change in total prostate-specific antigen concentrations over a period of time
pT	pathological stage (TNM with histopathological evaluation of tumor)
ROC	receiver-operating characteristic (curve)
scFv	single chain variable fragments of an antibody
thK2	total kallikrein-related peptidase 2
TNM	cancer staging classification method (tumor, nodes, metastasis)
tPSA	total prostate-specific antigen
tPSA(2C1)	immunoassay for measuring tPSA, Mab 2C1 as the tracer
TRUS	transrectal ultrasound
WHO	World Health Organization

ABSTRACT

Prostate-specific antigen (PSA) is a marker that is commonly used in estimating prostate cancer risk. Prostate cancer is usually a slowly progressing disease, which might not cause any symptoms whatsoever. Nevertheless, some cases of cancer are aggressive and need to be treated before they become life-threatening. However, the blood PSA concentration may rise also in benign prostate diseases and using a single total PSA (tPSA) measurement to guide the decision on further examinations leads to many unnecessary biopsies, over-detection, and overtreatment of indolent cancers which would not require treatment. Therefore, there is a need for markers that would better separate cancer from benign disorders, and would also predict cancer aggressiveness. The aim of this study was to evaluate whether intact and nicked forms of free PSA (fPSA-I and fPSA-N) or human kallikrein-related peptidase 2 (hK2) could serve as new tools in estimating prostate cancer risk.

First, the immunoassays for fPSA-I and free and total hK2 were optimized so that they would be less prone to assay interference caused by interfering factors present in some blood samples. The optimized assays were shown to work well and were used to study the marker concentrations in the clinical sample panels.

The marker levels were measured from preoperative blood samples of prostate cancer patients scheduled for radical prostatectomy. The association of the markers with the cancer stage and grade was studied. It was found that among all tested markers and their combinations especially the ratio of fPSA-N to tPSA and ratio of free PSA (fPSA) to tPSA were associated with both cancer stage and grade. They might be useful in predicting the cancer aggressiveness, but further follow-up studies are necessary to fully evaluate the significance of the markers in this clinical setting.

The markers tPSA, fPSA, fPSA-I and hK2 were combined in a statistical model which was previously shown to be able to reduce unnecessary biopsies when applied to large screening cohorts of men with elevated tPSA. The discriminative accuracy of this model was compared to models based on established clinical predictors in reference to biopsy outcome. The kallikrein model and the calculated fPSA-N concentrations (fPSA minus fPSA-I) correlated with the prostate volume and the model, when compared to the clinical models, predicted prostate cancer in biopsy equally well. Hence, the measurement of kallikreins in a blood sample could be used to replace the volume measurement which is time-consuming, needs instrumentation and skilled personnel and is an uncomfortable procedure. Overall, the model could simplify the estimation of prostate cancer risk.

Finally, as the fPSA-N seems to be an interesting new marker, a direct immunoassay for measuring fPSA-N concentrations was developed. The analytical performance was acceptable, but the rather complicated assay protocol needs to be improved until it can be used for measuring large sample panels.

In conclusion, by combining different markers it is possible to enhance the prostate cancer risk estimation and avoid unnecessary biopsies and adverse effects of over-detection and overtreatment.

TIIVISTELMÄ

Joka vuosi tuhannet miehet Suomessa ja muualla maailmassa sairastuvat eturauhassyöpään, joka on Suomessa ja monessa muussa länsimaassa miesten yleisin syöpä. Eturauhassyöpä aiheuttaa paitsi huolta ja tuskaa kantajalleen myös merkittävät kustannukset terveydenhuoltojärjestelmälle. Eturauhassyöpä on yleensä hitaasti etenevä, eivätkä kaikki syövät aiheuta koskaan oireita. Osa syövistä kuitenkin etenee nopeasti ja voi lopulta aiheuttaa jopa kuoleman. Näiden syöpien löytyminen mahdollisimman varhaisessa vaiheessa on tärkeää, jotta hoito olisi tehokasta.

Prostata-spesifinen antigeeni (PSA) on verenkierrossa oleva proteiini, jota käytetään yleisesti biomerkkiaineena eturauhassyövän riskin arvioinnissa. Veren PSA-taso voi kuitenkin nousta myös muissa eturauhasen sairauksissa. Jos yksittäisen kokonais-PSA-määrityksen (tPSA) perusteella päätetään jatkotutkimuksista, seurauksena on monien turhien koepalojen otto, oireettomien hitaasti kasvavien syöpien löytyminen (ylidiagnostiikka) sekä turha hoito (ylihoito). On siis olemassa tarve biomerkkiaineelle, joka auttaisi erottamaan syövän muista eturauhasen sairauksista ja toisaalta hitaasti kasvavat syövät aggressiivista syövistä. Tämän tutkimuksen tarkoituksena oli selvittää, voitaisiinko vapaan PSA:n (fPSA) eri muotoja (ehjä fPSA eli fPSA-I ja sisäisesti pilkkoutunut fPSA eli fPSA-N) tai ihmisen kallikreiinin kaltaista peptidaasi 2:ta (hK2) hyödyntää eturauhassyövän riskin arvioinnissa.

Immunomääritysten, joilla fPSA-I- ja hK2-määriä mitataan, todettiin olevan herkkiä verinäytteissä oleville erilaisille häiritseville tekijöille ja vaativan kehittämistä. Tehtyjen parannusten jälkeen määritykset toimivat hyvin, ja niitä voitiin käyttää kliinisten näytteiden biomerkkiainepitoisuuksien mittaamiseen.

Biomerkkiaineiden pitoisuudet mitattiin eturauhasen poistoleikkaukseen menossa olevilta eturauhassyöpäpotilailta ennen leikkausta otetuista verinäytteistä. Pitoisuuksia verrattiin syövän levinneisyysasteeseen ja pahanlaatuisuuteen. Biomerkkiaineista ja niiden suhteista erityisesti fPSA-N:n ja tPSA:n suhde sekä fPSA:n ja tPSA:n suhde olivat yhteydessä syövän levinneisyyteen ja pahanlaatuisuuteen. Nämä pitoisuudet ja suhteet saattaisivat auttaa syövän aggressiivisuuden arvioinnissa, mutta tarvitaan laajempia seurantatutkimuksia, jotta niiden todellinen kliininen arvo voidaan todeta.

Biomerkkiaineista tPSA, fPSA, fPSA-I ja hK2 on aikaisemmin yhdistetty tilastolliseen malliin, jonka avulla voitaisiin vähentää turhien koepalojen ottamista miehiltä, joilla on kohonnut tPSA-taso. Tässä väitöskirjatutkimuksessa edellä mainitun kallikreiinimallin kykyä ennustaa syövän läsnäolo koepaloissa verrattiin malleihin, joissa oli mukana nykykäytännön mukaisia kliinisiä tietoja. Kallikreiinimalli ja laskennallinen fPSA-N-pitoisuus (fPSA – fPSA-I = fPSA-N) korreloivat eturauhasen tilavuuden kanssa, ja kallikreiinimalli myös ennusti syövän läsnäolon koepaloissa yhtä hyvin kuin kliinisiä tietoja sisältävä malli. Täten verinäytteestä tehtävät biomerkkiainemääritykset voisivat korvata ultraäänen avulla tehtävän eturauhasen tilavuuden mittaamisen, joka vaatii erityislaitteiston ja osaavan henkilökunnan, ja joka voi olla myös epämukava toimenpide.

Kaiken kaikkiaan näiden biomerkkiaineiden mittaaminen voisi yksinkertaistaa eturauhassyövän riskin ennustamista.

Koska fPSA-N osoittautui kiinnostavaksi biomerkkiaineeksi, kehitettiin immunomääritys sen mittaamiseksi verinäytteistä suoraan sen sijaan, että pitoisuus olisi määritetty laskennallisesti muiden määritysten tuloksista. Määritys toimi hyvin, mutta sen suorittaminen on jossain määrin monimutkaista, joten määritys tarvitsee kehittämistä ennen kuin sillä voidaan mitata isoja näyte-eriä.

Yhteenvetona voidaan todeta, että yhdistämällä eri biomerkkiaineita on mahdollista parantaa eturauhassyövän riskin ennustamista. Näin vältetään turhien koepalojen ottamista ja vähennetään ylidiagnostiikasta ja ylihoidosta aiheutuvia haittoja.

1 INTRODUCTION

Every year thousands of men hear that they have prostate cancer, the most common cancer of men in Finland. It causes mental and physical suffering to these men and their families as well as significant costs to the healthcare system. In 2010 a total of 4719 men were diagnosed with prostate cancer in Finland which was 31.6% of all cancers diagnosed in men. Prostate cancer was the second most common cause of cancer deaths among men with 847 cases (13.8% of all cancer deaths) after the lung cancer, the cause of death in 1547 cases (25.2% of all cancer deaths). (Finnish Cancer Registry, 2012)

Prostate cancer is a slow growing disease and it rarely causes clinical symptoms. Many men die without ever knowing they had developed the cancer and most likely the actual cause of death is other than prostate cancer. The current question is which cases of prostate cancer should we find and treat and which ones do not cause any actual harm to the men. The following question is how should we proceed to find those cases of cancer: by carrying out age-group specific screening of the entire male population, by estimating the risk of each man with some kind of a statistical method or just by reacting to the symptoms once they occur?

Prostate-specific antigen (KLK3, PSA) has had an important role in finding cases of prostate cancer since the late 1980s. Large screening studies were started in the 1990s to find out how good PSA would be as a screening method and now that the first results have been reported the question still remains without an unambiguous answer (Andriole *et al.*, 2009; Schroder *et al.*, 2009). On one hand, it has become clear over the years that many men are diagnosed and also treated unnecessarily because many cases of indolent cancer are found by using PSA testing. On the other, there are definitely aggressive cases of cancer which need to be found as early as possible to treat the men, increase their life span and quality of life and finally prevent the cancer deaths.

In this thesis, PSA and its different forms in blood circulation as well as human kallikrein-related peptidase 2 (KLK2, hK2) were studied as possible biomarkers to improve the prostate cancer diagnostics. The two proteins are part of a family of 15 proteins for which a new nomenclature system was suggested in 2006 (Lundwall *et al.*, 2006). In this system the genes encoding each member of the family are called as kallikrein-related peptidases (*KLK*) followed by the number of the gene symbol, except for the first member which is still called kallikrein 1. The corresponding proteins are called with the same names except that the abbreviations are written in standard font to separate them from the genes which are written in italics. Despite the suggestion for nomenclature the old names for KLK3 and KLK2 are commonly used and for practical reasons in this thesis the old abbreviations PSA and hK2 are used instead of the suggested ones.

2 REVIEW OF THE LITERATURE

2.1 Prostate cancer

Prostate is a male secretory gland situated below the bladder and surrounding the urethra. Its biological function is to produce 20% of the liquid that forms the major part of semen. It consists of glandular structures which are surrounded by stromal structures. There are three distinct parts of the prostate: central, transition and peripheral zones.

2.1.1 Pathogenesis

Prostate cancer is both a biologically and clinically heterogeneous disease but it is often a slow growing type of cancer and many men have it without any symptoms during their life. It has been estimated that 30% of the men over 50 years and 70–80% of the men over 80 years of age have the cancer but only one out of ten of these cases of latent cancer develop into a clinically diagnosed cancer (Jemal *et al.*, 2005; Prostate Cancer: Current Care guideline, 2007). The highest rates of prostate cancer occur in the United States and Europe while the men in Asia suffer least of the prostate cancer (Hsing *et al.*, 2000).

Most cases of cancer (70%) occur in the peripheral zone of the prostate and they are adenocarcinomas which means that the cancer cells originate from the epithelium (Lee *et al.*, 2011). In the normal prostate's glandular structure the epithelial secretory cells are columnar, usually as a single-cell high layer, and form acini (round sacs or cavities surrounded by the cells) which have a basal cell layer to support the structure. The histological definition of prostate cancer involves the loss of the basal cell layer. Prostatic intraepithelial neoplasia (PIN) is considered as a premalignant transformation of the epithelial cells. The low grade PIN (LGPIN) may have more than one layer of epithelial cells but the basal cell layer is still intact. In high grade PIN (HGPIN) the basal cell layer is defined as discontinuous and these cells may be difficult to separate from actual well differentiated cancer cells. (Ayala and Ro, 2007)

In its early phases prostate cancer tumor occurs within the prostate capsule and it may be multifocal meaning that there are several separated tumors which may even be genetically different. If the prostate cancer grows outside of the prostate capsule it may spread to the seminal vesicles or bladder neck. If the cancer metastasizes further it may do so either via lymph or blood typically to bone. Both prostate and cancer cells are androgen regulated but often, in the late, metastasized phases of the cancer progression, cancer cells lose their sensitivity to androgens.

It seems that environmental factors may have a stronger effect on prostate cancer development than inherited factors (Lichtenstein *et al.*, 2002; Lichtenstein *et al.*, 2000). About 2–3% of prostate cancers are inherited and approximately 20% appear within certain families (Prostate Cancer: Current Care guideline, 2007). Especially a diet with increased fat and red meat consumption seems to be related to an increased risk of prostate cancer (Giovannucci *et al.*, 1993; Whittemore *et al.*, 1995). On the other hand a diet with a high intake of tomatoes (or lycopene from other sources) or soy may protect from the

prostate cancer (Badger *et al.*, 2005; Chen *et al.*, 2001; Etminan *et al.*, 2004; Gann *et al.*, 1999). The effect of diet and environmental factors is probably the key factors in the rise of the prostate cancer risk when men from Asia or other low-risk countries move to North America (Marks *et al.*, 2004; Shimizu *et al.*, 1991; Watanabe *et al.*, 2000).

2.1.2 Diagnosis, staging and grading

Diagnosis

Prostate cancer may not cause any symptoms in the early phase but after progressing to some extent the symptoms may include urinating difficulties, blood secretion in urine or bone pain in the cases of already metastasized cancer. When prostate cancer is suspected the clinical examination involves serum PSA measurement (in Finland both total and free PSA), palpation through rectum which is called digital rectal examination (DRE), and later also transrectal ultrasound (TRUS) can be used. Diagnosis is always made by histological findings from a prostate biopsy tissue sample. The biopsies (6–12 or even up to 21) are taken with a needle under TRUS guidance through rectum (Chun *et al.*, 2010; Prostate Cancer: Current Care guideline, 2007). Prostate cancer may also be found incidentally in prostate tissue removed during transurethral resection of the prostate performed to relieve the symptoms of benign growth of the prostate.

Prostate cancer symptoms may be similar to symptoms of other diseases of prostate so an essential part of prostate cancer diagnosis is discrimination of benign conditions from cancer. Most common benign diseases are benign prostatic hyperplasia (BPH) and prostatitis.

In BPH the transition zone of the prostate has grown so much that it causes symptoms, most often urinating difficulties. This is caused by the overgrown prostate tissue which is squeezing the urethra. The prostate continues to grow throughout a man's life so BPH is very common. It has been estimated that approximately 80% of the men over 60 years of age have histological signs of BPH and 40% suffer from the symptoms (Garraway *et al.*, 1991; Sagnier *et al.*, 1996; Sanda *et al.*, 1997). BPH may also cause the serum PSA to rise.

Prostatitis may be caused by a bacterial infection either as an acute or chronic condition. Coliform, enterococci and staphylococci bacteria are common causes and the infection can usually be cured with antibiotics (Sharp *et al.*, 2010). Infectious prostatitis may occur after taking a prostate biopsy (Loeb *et al.*, 2011; Nam *et al.*, 2010). It is also possible that prostatitis is caused by inflammation without any signs of bacterial infection. The disease is called chronic pelvic pain syndrome, and its etiology is not known. This syndrome can also exist without inflammatory signs and it may also cause the serum PSA to rise. Some prostatitis cases are found only in histological samples taken due to some other urological conditions. (Sharp *et al.*, 2010.)

Prostate cancer fulfills many characteristics of a disease which could be screened across the male population. It is a major health issue, there is a screening method with which it can be found in an early stage and it is curable at its early stage. The most common

screening method is the PSA measurement, but also DRE or TRUS can be used for screening. The screening of prostate cancer seems to lead to some reduction in mortality with the cost of over-detection and overtreatment (Schroder *et al.*, 2009; Schroder *et al.*, 2012). Over-detection means that many cases of cancer that would not cause any harm to the men are detected and thus many unnecessary biopsies are taken causing unnecessary anxiety to the men. Over-detection may lead to overtreatment which means that cases of cancer that could be followed without treatment are actually treated with aggressive methods which may cause more serious side-effects than just doing nothing. In addition to this, there is no information on the cost-benefit ratio of the screening or how screening affects the quality of life. Thus, the population-based screening is not currently recommended in Finland (Prostate Cancer: Current Care guideline, 2007). The effect of PSA-based screening is discussed in more detail in chapter 2.2.2.

Stage

The extent, or spread, of the prostate cancer is defined by the TNM staging classification of American Joint Committee on Cancer and the International Union against Cancer (**Table 1**). The classification system is based on the cancer status of the prostate gland itself (T), lymph nodes (N) and other metastases (M). Organ-confined cancers are not growing outside of the prostate capsule and non-organ-confined cancers have grown through the capsule. Pelvic lymph nodes are usually the first lymph nodes where the prostate cancer metastasizes and the very advanced cancer most often send metastases to the bones.

The histopathological staging of the prostate can be done after removal of the prostate (radical prostatectomy). Essentially the same definitions are used for both clinical (cTNM, **Table 1**) and pathological (pTNM) staging. The pathological staging may provide some additional information, for example whether there is a possibility that some extracapsular parts of the tumor have not been successfully removed.

Grade

Prostate cancer's progression can be estimated by grading the histological tissue sample. The grading classifies the tissue patterns by the morphology of the tissue structures or the cell morphology. Usually, the more differentiated the cells and other structures are the less aggressive the cancer is. The loss of glandular structure and deformed cells are usually a sign of potentially aggressive cancer which may end up sending metastases to other parts of the body.

Gleason grading is a commonly used method to evaluate prostate cancer tissue. It was developed by Dr. Donald Gleason with The Veterans Administration Cooperative Research Group in 1966 (Gleason, 1966; Gleason and Mellinger, 1974). It has been updated since the first publication and the latest version was published in 2005 (Epstein, 2010; Epstein *et al.*, 2005). The Gleason grading system is based on the overall appearance of the tissue and the individual cell morphology does not play an important role. It takes into account that the cancer cells do not progress uniformly and that there may be several different types of cancer tissue in one prostate. The Gleason grade is given

in numbers between 1 and 5 so that the most differentiated tissue is given grade 1 and the least differentiated tissue is grade 5 (see **Figure 1**). The two predominant patterns, primary and secondary, are graded, and the grades are then added up to get the Gleason score which can vary between 2 and 10.

Table 1. The 1997 TNM cancer staging classification of the American Joint Committee on Cancer and the International Union against Cancer for prostate cancer. TNM stands for Tumor, Nodes, Metastases. The classification system was updated in 2002 and 2009 but the 1997 version is shown here because it was used in the current study (Edge *et al.*, 2010; Greene *et al.*, 2002; Sobin LH, 1997).

Tumor	**Sub-stage**	**Definition**
TX		Primary tumor cannot be assessed
T0		No evidence of primary tumor
T1		Clinically inapparent tumor, not palpable or visible by imaging
	T1a	Incidental histologic finding in < 5% of resected tissue
	T1b	Incidental histologic finding in > 5% of resected tissue
	T1c	Tumor identified by needle biopsy (e.g. due to elevated PSA)
T2		Confined within the prostate
	T2a	Tumor involves one lobe
	T2b	Tumor involves both lobes
T3		Tumor extends through the prostate capsule but has not spread to other organs
	T3a	Extracapsular extension (unilateral or bilateral)
	T3b	Tumor invades seminal vesicles
T4		Tumor is fixed or invades adjacent structures other than seminal vesicles: bladder neck, external sphincter, rectum, levator muscles and/or pelvic wall

Nodes	**Sub-stage**	**Definition**
NX		Regional lymph nodes cannot be assessed
N0		No regional lymph node metastasis
N1		Regional lymph node metastasis

Metastasis	**Sub-stage**	**Definition**
MX		Distant metastasis cannot be assessed
M0		No distant metastasis
M1		Distant metastasis
	M1a	Non-regional lymph node metastasis
	M1b	Bone metastasis
	M1c	Metastasis at other sites

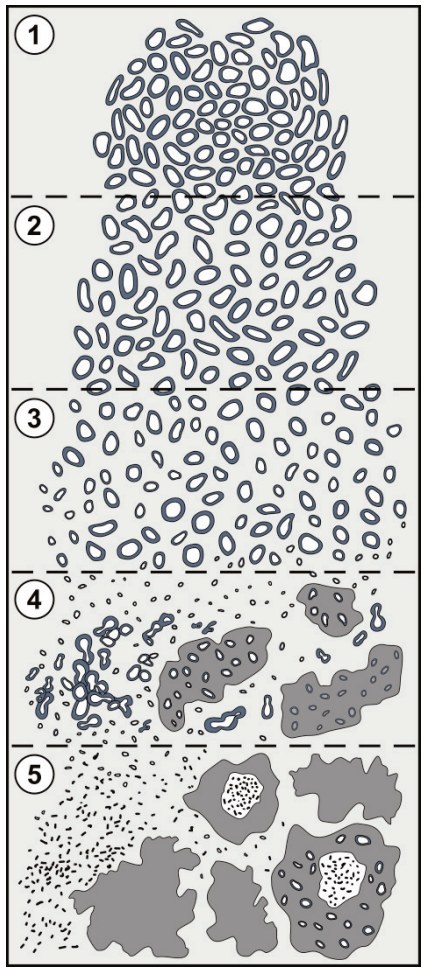

Figure 1. The Gleason grading of prostate cancer (Modified from Epstein *et al.*, 2005 by Johanna Vuojola).

Another type of grading has been named as World Health Organization (WHO) grade which was developed by Dr. F. K. Mostofi in 1975 (Mostofi, 1975). In this grading system the cell morphology is also taken into account. The evaluated characteristics include increased cellularity, nuclear crowding, disturbance of cellular polarity, failure of differentiation of epithelial cells from the basal layer of epithelium, polymorphism, irregularity in cell size, variations of shape and chromatin pattern of the nuclei, displaced or abnormal mitotic figures and giant cells. The grading is given in numbers from 1 to 3 in which the grade 1 is the least anaplastic (i.e. the cells are more differentiated) and the grade 3 is the most anaplastic type (i.e. the cells are not differentiated).

2.1.3 Treatment and prognosis

The treatment of prostate cancer is currently efficient for localized cancer, especially for organ-confined tumors. The low stage, low grade cancers can be left without treatment under so called watchful waiting or active surveillance, especially if the patient is over 70 years old (or the expected lifetime is less than 10 years for other reasons than prostate cancer). The localized cancers are most often treated with removal of the prostate (radical prostatectomy) or with different forms of radiation therapy. The prognosis is good, the 10-year survival rate is 90–94% (Albertsen *et al.*, 2007; Lu-Yao and Yao, 1997).

The treatment may have adverse effects like complications during the surgery and post-treatment incontinence or impotency. The rates for retaining continence and potency vary between different treatment techniques and for example between different surgeons. Generally 58–97% of the radical prostatectomy patients retain continence and 31–94% retain potency. The overall complication rate during radical prostatectomy operations is approximately 10%. (Coelho *et al.*, 2010.) Radiation therapy may also cause side effects to a significant number of patients (up to 20 %) (Prostate Cancer: Current Care guideline, 2007). Due to these possible adverse effects treatment of an indolent cancer may cause more harm than benefit to the patient.

If the cancer has metastasized, it may be treated with both radical prostatectomy or radiation therapy and castration by removal of testes (orchiectomy) or chemically preventing androgen secretion. As the prostate cells are under androgen regulation this kind of treatment may prolong the patient's life by 2–3 years but if the cancer turns into a hormone-resistant type the lifetime expectancy is only from half to one and half years (Berry *et al.*, 2006; Tannock *et al.*, 1996).

2.2 Free prostate-specific antigen forms in prostate cancer diagnostics

2.2.1 Structure and function of prostate-specific antigen (PSA)

PSA (KLK3) is a serine protease produced in the prostate epithelial cells as a 261 amino acid preproPSA. The 17 amino acid prepeptide is cleaved in endoplasmic reticulum and the resulting proPSA is transported to the plasma membrane (McCormack *et al.*, 1995). The 7 amino acid propeptide is cleaved to produce the enzymatically active PSA which has chymotrypsin-like substrate specificity. PSA is secreted to the lumen of prostatic ducts and eventually it forms part of the seminal fluid. PSA is activated by hK2 (KLK2), KLK4, trypsin and other kallikreins (Kumar *et al.*, 1997; Lovgren *et al.*, 1997; Takayama *et al.*, 2001a; Takayama *et al.*, 1997; Takayama *et al.*, 2001b; Yoon *et al.*, 2007) and its main biological function is to cleave gel-forming proteins in semen to liquefy the semen and thus enable spermatozoa motility (Lilja, 1985; Lilja *et al.*, 1987; Malm *et al.*, 2000; McGee and Herr, 1988). PSA has also many putative functions which relate to, for example, enhancing the invasion and proliferation of tumor cells (Lawrence *et al.*, 2010). The five disulfide bonds hold the molecule together even if it would be inactivated through internal peptide bond cleavages.

The molecular mass of PSA polypeptide is 26 079 Da. PSA has one glycosylation site at Asn45 and the carbohydrate moiety increases the mass with approximately 2300 Da. Therefore, depending on the structure of the moiety, the overall mass of PSA is around 28 400 Da (Belanger *et al.*, 1995; Mattsson *et al.*, 2008). PSA protease function is partly regulated by complex formation with its inhibitors of which α_1-antichymotrypsin (ACT, SERPINA3) and α_2-macroglobulin (A2M), α_1-antitrypsin (AT or API, SERPINA1) and protein C inhibitor (PCI, SERPINA5) are the most important (Christensson *et al.*, 1990; Christensson and Lilja, 1994; Espana *et al.*, 1991; Stenman *et al.*, 1991; Zhang *et al.*, 1997). Most of the PSA in the prostate tissue and seminal plasma is in free and active (noncomplexed) form (Christensson and Lilja, 1994; Denmeade *et al.*, 1997; Espana *et al.*, 1993; Mikolajczyk *et al.*, 1997a; Mikolajczyk *et al.*, 2000b). The PSA found in blood circulation is mostly (65–95%) complexed but the inactive forms (5–35%), including proPSA and internally cleaved forms, do not form complexes (Lilja *et al.*, 1991; Stenman *et al.*, 1991; Zhang *et al.*, 1999).

PSA is almost exclusively expressed in the prostate even though it has been detected in small quantities in other tissues and body fluids (Shaw and Diamandis, 2007). The epithelial cells secreting PSA have a strong polarity towards the prostatic ducts and only a very small proportion of the PSA is able to escape into blood circulation. The structural changes in tissue caused by different disease states cause more PSA to enter the blood stream which is the rationale for using PSA as a biomarker. As infections, inflammations and benign growth (BPH) of the prostate can cause more PSA to escape into the blood as well, PSA is not a prostate cancer specific marker but rather an indicator of an abnormal prostate status.

2.2.2 Circulating PSA as a marker for prostate cancer

Since the first proposal of using PSA for cancer detection (Papsidero *et al.*, 1980) it has become the most important tumor marker – despite its lack of specificity. The ways of using PSA measurements differ greatly between different countries. In the United States and Canada the PSA measurements are very common and they are recommended by different organizations but in slightly different way (Greene *et al.*, 2009; Heidenreich *et al.*, 2011; Kawachi *et al.*, 2010; Wolf *et al.*, 2010). The general trend seems to encourage the patient and doctor to discuss all possible consequences of performing the assay and then base the decision on the discussion. As new information emerges from the studies the recommendations have been changing since the widely accepted suggestion to use 4 ng/mL PSA cut-off for further examination by Catalona *et al.* (Catalona *et al.*, 1991).

Recently, two large population-based randomized prostate cancer screening studies have reported results which were somewhat controversial. The North American Prostate, Lung, Colorectal, and Ovarian (PLCO) Cancer Screening Trial reported that PSA-based screening did not affect prostate cancer mortality after a 7 to 10 year follow-up (Andriole *et al.*, 2009) while the European Randomized study of Screening for Prostate Cancer (ERSPC) reported a 20–30% reduction in prostate cancer deaths but also a need for large number of men screened (> 1000 men) and diagnosed (37 men) with cancer to prevent one prostate cancer death (Roobol *et al.*, 2009b; Schroder *et al.*, 2009; Schroder *et al.*, 2012).

In other words, there is a big risk for over-detection (overdiagnosis) which may lead to overtreatment: while only a part of the diagnosed cases of cancer are actually life threatening it may be difficult to predict which cases are indolent and which need to be treated. Furthermore, many men do not want to live with the cancer and want to be treated rather than rely on active surveillance of the cancer. In addition to causing costs to the healthcare system, the side-effects of an unnecessary treatment (for example urinary problems or impotence after removal of the prostate) may turn out to be worse than doing nothing. Another side of the procedure is the false-positive rate in the screening. In Finland, approximately 7% of the screened men had a PSA value above the screening cut-off but no cancer was found in the following biopsy, and when attending several rounds of screening as many as one in every eight men may have a false-positive result (Kilpelainen *et al.*, 2010). This emphasizes the need for better tools for estimating the cancer risk of an individual man.

In Europe PSA is used less than in the North America and it is recommended in many countries only for symptomatic patients. The degree of so called opportunistic screening for prostate cancer differs between countries as well. Opportunistic screening means that patients or clinicians want to measure PSA values even though there may not be any direct evidence for the need for it.

In Finland, The Current Care guideline suggests PSA measurements only for symptomatic patients (Prostate Cancer: Current Care guideline, 2007). Total PSA (tPSA) concentrations, comprising both complexed and free PSA forms, rise with age and, therefore, age-specific reference ranges are used (**Table 2**). For patients with tPSA values within the range of 2.5–10 ng/mL the measurement of the free PSA (fPSA) is recommended because the ratio of fPSA and tPSA (F/T PSA) is generally lower in prostate cancer patients and calculating the F/T PSA ratio is likely to help in deciding whether a biopsy is necessary (**Table 3**). In addition to using tPSA as an aid in cancer diagnostics it is widely used for the follow-up of the radical prostatectomy patients. The PSA concentration in their blood circulation should remain low and a rising concentration is usually a sign of a recurrent cancer.

Table 2. Age-specific reference ranges for total PSA (tPSA) used in Finland. µg/L = ng/mL (Catalona *et al.*, 1994; Prostate Cancer: Current Care guideline, 2007; Thompson *et al.*, 2004)

Age (years)	Serum tPSA (µg/L)
40–49	< 2.5
50–59	< 3.5
60–69	< 4.5
70–79	< 6.5

Table 3. PSA concentrations and prostate cancer risk. µg/L = ng/mL (Catalona *et al.*, 1994; Prostate Cancer: Current Care guideline, 2007; Thompson *et al.*, 2004)

PSA concentration in serum or plasma	**Risk of prostate cancer**
tPSA µg/L	%
0–2	1
2–4	15
4–10	25
> 10	> 50
F/T PSA (%) when tPSA 4–10 µg/L	
0–10	56
10–15	28
15–20	20
20–25	16
> 25	8

F/T PSA has been shown to help in discriminating prostate cancer from BPH in many studies since the first publications of serum fPSA (Lilja *et al.*, 1991; Stenman *et al.*, 1991). The F/T PSA has helped in discriminating men with and without cancer with a tPSA within the range of 2.5–10 ng/mL but it seems that the discriminative power is decreased in men with a tPSA of 2.5–4 ng/mL (Bangma *et al.*, 1997; Catalona *et al.*, 1998; Raaijmakers *et al.*, 2004; Recker *et al.*, 2001). However, an F/T PSA < 15% in men with a tPSA < 3 ng/mL may predict future cancer (Finne *et al.*, 2008). In addition, F/T PSA seems to help in predicting cancer aggressiveness (Bjork *et al.*, 1999; Raaijmakers *et al.*, 2004; Shariat *et al.*, 2006; Southwick *et al.*, 1999). fPSA is also a significant factor, together with tPSA, in many statistical models predicting prostate cancer risk (Chun *et al.*, 2007; Finne *et al.*, 2004; Steuber *et al.*, 2007a; Vickers *et al.*, 2008). The models and risk calculators are discussed in more detail in chapter 2.5.

In addition to F/T PSA ratio, PSA velocity and density have also been suggested to help in assessing the prostate cancer risk. PSA velocity (PSAV) is calculated from PSA measurements made over a period of time to see whether the PSA concentration is rising, which can be a sign of developing disease (BPH or prostate cancer) (Carter *et al.*, 1992; Loeb *et al.*, 2007; Meeks *et al.*, 2008). According to the Finnish Current Care guideline further examination is recommended when a PSAV exceeds 0.75 ng/mL per year (based on three measurements made within a time period of at least one year) in a man with tPSA within the range of 4–10 ng/mL. However, PSAV may not exceed the significance of F/T PSA in a screening setting as has been suggested recently (Schroder *et al.*, 2006; Ulmert *et al.*, 2008; Vickers *et al.*, 2009).

PSA density (PSAD) is the serum (total) PSA concentration divided by the prostate volume. As prostate volume, as well as serum PSA, increases with age and in BPH it has

been suggested that large amounts of PSA leaking into blood circulation from a small prostate, thus increasing the ratio of PSAD, would be an indication of cancer (Epstein *et al.*, 1994; Freedland *et al.*, 2005; Kranse *et al.*, 1999; Roehrborn *et al.*, 1999a; Roehrborn *et al.*, 1999b; Seaman *et al.*, 1993). Prostate volume and PSAD have provided additional discriminative power in some statistical models predicting cancer risk or cancer pathology (Newton *et al.*, 2010; Radwan *et al.*, 2007; Roobol *et al.*, 2012). More aggressive types of cancer have been found in small prostates but there is debate about whether this is caused by the biology of the cancer or whether it is due only to PSA measurement or biopsy regime characteristics (Briganti *et al.*, 2007; Newton *et al.*, 2010; Ngo *et al.*, 2012; van Leeuwen *et al.*, 2009). However, the volume measurement (or estimation) requires either a digital rectal examination (Roobol *et al.*, 2012) or transrectal ultrasound measurement which should be performed by a urologist and are considered to be invasive procedures.

The current commercial immunoassays for tPSA measure free and complexed forms (mostly PSA-ACT) but not PSA bound to A2M which is not immunoreactive in conventional immunoassays. The assays are expected to recognize both forms equally (equimolar recognition) and the manufacturers should inform the users on how the assays are calibrated. The equimolarity and standardization issues are discussed in more detail in chapter 2.4.2. A few assays for measuring complexed PSA (cPSA) exist but it seems that its clinical significance is approximately the same as that of fPSA measurement (Leinonen *et al.*, 1993). Most manufacturers offer fPSA assays which are generally expected to recognize all noncomplexed PSA forms. There are also immunoassays which are specific for certain subforms of fPSA.

The first studies investigating the different forms of free PSA in serum were made in the mid-1990s. Huber *et al*. (1995) found that the pI values of PSA forms in the serum of prostate cancer patients were higher than in the serum of BPH patients and they speculated that different glycosylation would explain this difference (Huber *et al.*, 1995). A few years later the precursor forms, or proPSA, as well as internally cleaved forms were found in the serum of prostate cancer patients (Mikolajczyk *et al.*, 1997a; Noldus *et al.*, 1997). Soon it was reported that the proPSA and inactive intact mature forms were more abundant in the serum of prostate cancer patients while the serum of BPH patients had more internally cleaved forms (Charrier *et al.*, 1999; Hilz *et al.*, 1999; Qian *et al.*, 1997). These findings were supported by observations of different PSA forms in prostate tissue. Internally cleaved, or nicked, PSA was found in BPH nodules and proPSA forms were more often present in cancer tissue than in benign transition zone tissue (Chen *et al.*, 1997; Mikolajczyk *et al.*, 2000a). The different fPSA forms and their clinical use are described in more detail in the **Figure 2** and in the following chapters.

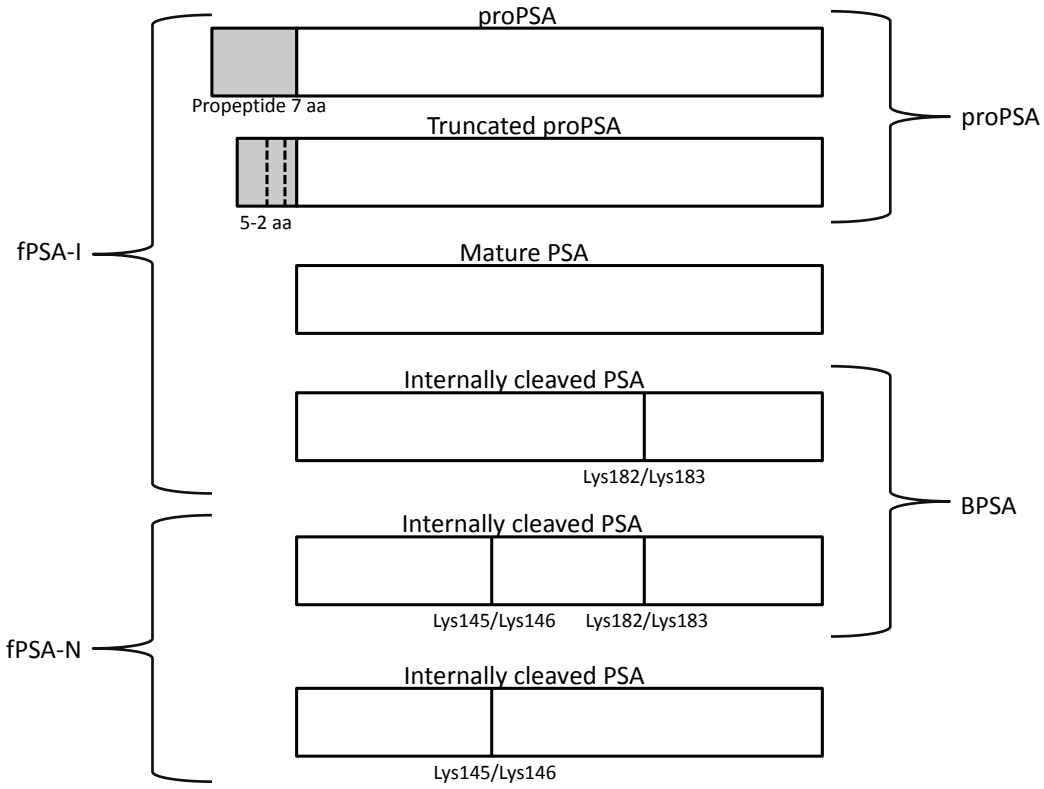

Figure 2. fPSA subforms and the immunoassays recognizing them. PSA is presented as the bar in which the propeptide is gray, propeptide cleavage sites are marked with dashed vertical lines and internal cleavage sites with solid vertical lines. The amino acids at internal cleavage sites are indicated. The forms detected by different immunoassays are indicated on the left (fPSA-I and fPSA-N) and on the right (proPSA and BPSA).

2.2.3 Precursor forms of PSA (proPSA)

The pro-sequence of PSA consists of seven N-terminal amino acids which are cleaved by hK2, trypsin or other proteases to form the enzymatically active mature PSA. The process is very rapid and efficient as no proPSA have been found in seminal plasma (Mikolajczyk *et al.*, 2001). In the prostate tissue and in the serum of prostate cancer patients PSA forms with different number of pro-sequence amino acids have been found. The number of the remaining amino acids is depicted by a negative number: [-7] referring to the full length pro-sequence and [-1] for the last amino acid before the mature PSA sequence starts. Most common proPSA forms are [-7], [-5], [-4] and [-2] while the forms [-6] and [-3] have not been found (Peter *et al.*, 2001). All proPSA forms are inactive but the [-5] and [-4] proPSA forms can be activated with hK2 or trypsin while the [-2]proPSA cannot (Mikolajczyk *et al.*, 2001). This probably enhances [-2]proPSA stability both in the prostate tissue and in the blood circulation making it a potential marker for prostate cancer (Chan *et al.*, 2003; Khan *et al.*, 2003; Mikolajczyk *et al.*, 2001).

Immunoassays recognizing different proPSA forms have been developed. An assay that measures [-7] and [-5] forms simultaneously, i.e. [-5,-7]proPSA assay, as well as assays for [-4] and [-2]proPSA forms have been developed by Hybritech Inc. (San Diego, USA), a Beckman Coulter subsidiary (Khan *et al.*, 2003; Mikolajczyk *et al.*, 2004; Mikolajczyk *et al.*, 2001; Sokoll *et al.*, 2003; Sokoll *et al.*, 2008). The assays have minimal cross-reactivity with other proPSA forms. Another assay for [-5,-7]proPSA was developed by Humboldt University (Berlin, Germany) in collaboration with Roche Diagnostics (Penzberg, Germany) (Bangma *et al.*, 2004; Hösel *et al.*, 2002; Lein *et al.*, 2005).

Several studies investigating the clinical potential of different proPSA forms have been conducted. Most of them have been rather small-scale and preliminary in nature (Bangma *et al.*, 2004; de Vries *et al.*, 2005; Khan *et al.*, 2003; Khan *et al.*, 2004; Mikolajczyk *et al.*, 2004; Naya *et al.*, 2004; Naya *et al.*, 2005; Sokoll *et al.*, 2003; Sokoll *et al.*, 2008). In a larger study (n = 1091) Catalona *et al*. found that using the ratio of summed concentrations of proPSA forms to fPSA concentration (%pPSA) improved the specificity of cancer detection over F/T PSA in men with a tPSA range of 2–10 ng/mL and that higher [-2]proPSA/fPSA ratios were associated with more aggressive cancer in cancer patients (Catalona *et al.*, 2003; Catalona *et al.*, 2004).

The results from studies measuring the [-5,-7]proPSA in cancer patients have not showed that this form would have clinical significance over F/T PSA (Bangma *et al.*, 2004; Catalona *et al.*, 2003; Lein *et al.*, 2005; Stephan *et al.*, 2006c) despite some contradictory data (Miyakubo *et al.*, 2009; Stephan *et al.*, 2007). Therefore, the recent studies have concentrated on the clinical utility of [-2]proPSA.

A common observation in most of the studies is that none of the proPSA forms is clinically useful as a single marker but rather in combination with either fPSA or tPSA (or both). The median concentration of [-2]proPSA in serum is approximately 14 pg/mL ranging from 1 pg/mL to nearly 100 pg/mL. The [-2]proPSA/fPSA ratio is approximately 1.5% ranging from under 0.5% to 6% and the ratio tends to be higher in cancer patients than in men without cancer (Guazzoni *et al.*, 2011; Sokoll *et al.*, 2010). Recently, more evidence of the true clinical value of [-2]proPSA has accumulated after the launch of the automated p2PSA immunoassay by Beckman Coulter. The studies consistently show that [-2]proPSA has additional value in predicting prostate cancer risk at initial biopsy and it could help in reducing unnecessary biopsies either as a ratio to fPSA or in a mathematical formula called "Beckman Coulter prostate health index" or phi. In the formula the concentration of [-2]proPSA has been combined with fPSA and tPSA concentrations. The phi is discussed in more detail in chapter 2.5.1. (Catalona *et al.*, 2011; Guazzoni *et al.*, 2012; Guazzoni *et al.*, 2011; Isharwal *et al.*, 2011; Jansen *et al.*, 2010; Le *et al.*, 2010; Sokoll *et al.*, 2010; Stephan *et al.*, 2009b)

More controversial data has been reported regarding the value of [-2]proPSA in predicting the aggressiveness of the prostate cancer. For example, Sokoll *et al*. reported [-2]proPSA/fPSA to be related with cancer aggressiveness in 245 prostate cancer patients (Sokoll *et al.*, 2010) but Jansen *et al*. state that it has limited additional value in identifying aggressive prostate cancer in 756 studied men of whom 400 were cancer cases

(Jansen *et al.*, 2010). Catalona *et al.* and Guazzoni *et al*. have reported the ability of either phi or [-2]proPSA/fPSA to predict high-grade cancer (Catalona *et al.*, 2011; Guazzoni *et al.*, 2012) even though Guazzoni *et al*. were more cautious in their conclusions regarding the relationship. In their earlier study of 268 men they found [-2]proPSA/fPSA to correlate with Gleason score but unable to improve the prediction of Gleason score of the prostate cancer in the biopsy (Guazzoni *et al.*, 2011). In the artificial neural network model created by Stephan *et al*. the inclusion of [-2]proPSA into the model improved prostate cancer detection and could replace prostate volume and DRE information in it (Stephan *et al.*, 2009b). Similar results were reported by Guazzoni *et al*. (2011) who reported the predictive accuracy of [-2]proPSA/fPSA and phi to exceed, for example, that of PSA density (see chapter 2.2.2).

2.2.4 Intact free PSA (fPSA-I)

The intact free PSA, or fPSA-I, is a PSA form which does not have an internal cleavage at the Lys145/Lys146 (Nurmikko *et al.*, 2000). The fPSA-I immunoassay is based on a unique antibody 4D4 (or 5C3) which does not recognize the PSA with cleavage at this site. The cleavage may also occur at Lys146/Leu147 (Chen *et al.*, 1997; Vegvari *et al.*, 2012) which is not recognized by the 4D4 either. For simplicity, in this thesis the site Lys145/Lys146 is used to refer to both possible sites. The cleavage at Lys145/Lys146 is probably the most common nicking site with or without other cleavages at Arg85/Phe86 or Lys182/Ser183 (Christensson *et al.*, 1990; Linton *et al.*, 2003; Mattsson *et al.*, 2008; Noldus *et al.*, 1997). The immunoassay recognizes both proPSA and mature PSA which may be in either inactive (Christensson *et al.*, 1990; Zhang *et al.*, 1995) or active form. Active PSA is found in very low amounts (~3% of fPSA) in the circulation (Niemelä *et al.*, 2002). As it is possible that, for example, cleavage at Lys182/Ser183 exists without a cleavage at Lys145/Lys146 (Linton *et al.*, 2003) the fPSA-I fraction is not fully intact PSA (i.e. PSA without any internal cleavages) but it is not clear whether the proportion of PSA with nicks somewhere else but not at Lys145/Lys146 is clinically significant. In seminal plasma the proportion of PSA with cleavage at Lys182/Ser183 but without one at Lys145/Lys146 is estimated to be approximately 10% so the proportion of it in blood circulation is likely to remain below or rise to 10% at the most (Linton *et al.*, 2003).

The first clinical studies assessing the value of fPSA-I suggested that the fPSA-I/fPSA ratio (I/F PSA) would help in discriminating men with and without cancer or benign disease (Niemelä *et al.*, 2002; Nurmikko *et al.*, 2001). The average serum fPSAI concentration is generally between 0.4 and 0.6 ng/mL and the short-term intra-individual variation of fPSA-I seems to be rather small (Christensson *et al.*, 2011). The change in the fPSA-I concentrations over a longer time period, i.e. fPSA-I velocity, is unlikely to facilitate predicting prostate cancer in biopsy (Vickers *et al.*, 2009).

The fPSA-I concentration as a single marker has not been significant in predicting the presence of prostate cancer. fPSA-I amounts to approximately 40–60% of fPSA and its ratio to fPSA is generally higher in cancer patients than in men without cancer (Gupta *et al.*, 2010; Niemelä *et al.*, 2002; Nurmikko *et al.*, 2001; Vickers *et al.*, 2010a; Vickers *et al.*, 2008). In a study of Steuber *et al*. (2005) fPSA-I concentration was not predictive of

prostate volume but it seemed to be associated with cancer volume despite the lack of statistical significance (Steuber *et al.*, 2005).

Later studies involved examination of large screening cohorts participating the European Randomized study of Screening for Prostate Cancer (ERSPC). Based on 740 screened men with elevated tPSA in Gothenburg, Sweden, a statistical model including fPSA-I as well as age, tPSA, fPSA and hK2 was developed for predicting the outcome of the initial biopsy. The model was able to significantly reduce the number of unnecessary biopsies, the reduction being 57% if a 20% risk of cancer would have been used as an indication for biopsy (Vickers *et al.*, 2008). Later studies confirmed these results in the previously unscreened men (Vickers *et al.*, 2010a) but also in recently screened men (Vickers *et al.*, 2010b; Vickers *et al.*, 2010c). fPSA-I is an essential part of the panel as demonstrated by the decrease in the predictive accuracy when fPSA-I is removed (Vickers *et al.*, 2011). The kallikrein panel is described and discussed in more detail in chapter 2.5.1.

2.2.5 Nicked free PSA (fPSA-N)

Nicked free PSA is defined as the fraction of free PSA containing the internal cleavage at Lys145/Lys146, thus being the fraction which is unrecognizable by the antibody 4D4 (or 5C3). As discussed in the previous chapter regarding fPSA-I (2.2.4) this cleavage may be the only one but it may also exist together with other internal cleavages. In various studies the fPSA-N concentrations have been calculated by subtracting fPSA-I concentrations from fPSA concentrations because there has not been a direct fPSA-N immunoassay available. A prototype of a direct fPSA-N assay was developed by Pauliina Niemelä in her doctoral thesis (Niemelä, 2002) and the assay was further optimized and published (IV) during this study.

BPH nodules and hyperplastic tissue in general contain more internally cleaved forms of PSA than proPSA forms or mature inactive PSA (Chen *et al.*, 1997; Mikolajczyk *et al.*, 2000b). The biological reason for this is currently unknown but it is thought that PSA is exposed to different proteases in the prostate tissue before diffusion to blood circulation.

The clinical value of fPSA-N seems to be in discriminating BPH patients from prostate cancer patients. Especially, the ratio of fPSA-N to tPSA (N/T PSA) has been significant in predicting the presence of benign enlargement of the prostate (Niemelä *et al.*, 2002; Nurmikko *et al.*, 2001; Steuber *et al.*, 2002). Ever since the first studies it has seemed evident that fPSA-N values as such are not of clinical value for individual patients but in a statistical combination with tPSA and fPSA the value might be much greater (Steuber *et al.*, 2005; Steuber *et al.*, 2002). As fPSA-N concentrations have been calculated from fPSA and fPSA-I concentrations either fPSA-N or fPSA-I has usually been excluded from the models to avoid problems that might be caused by their strong correlation with each other, or multicollinearity. In studies by Steuber *et al*. (2005 and 2007) fPSA-N was used in multivariate models. First, fPSA-N was found to be a significant predictor of prostate transition zone volume which is the common site for benign enlargement of the prostate (Steuber *et al.*, 2005). In the second study (Steuber *et al.*, 2007a), N/T PSA was found to be a strong univariate predictor of initial biopsy outcome, and fPSA-N was chosen into a

multivariate model with tPSA, fPSA and different forms of soluble urokinase plasminogen activator receptor (suPAR). fPSA-N was not an independent predictor in the model but provided additional diagnostic information. Also, it has been shown that the change in the fPSA-N concentrations over time, i.e. fPSA-N velocity, is not predictive of prostate cancer (Vickers *et al.*, 2009). In the same study, a logistic regression model including age, tPSA and fPSA-N had a better diagnostic performance (in terms of AUC, see chapter 2.5.1 for explanation) than age and tPSA combined with either fPSA, F/T PSA, fPSA-I or hK2.

2.2.6 Benign PSA (BPSA)

Benign PSA (BPSA) has a distinctive internal cleavage at Lys182/Ser183 which occurs most often together with a cleavage at Lys145/Lys146 as well as other cleavages. A fraction of BPSA (10–30%) may be without the cleavage at Lys145/Lys146 as discussed in chapter 2.2.4. BPSA was found in BPH nodules in transition zone of the prostate (Mikolajczyk *et al.*, 2000b) and later, after development of an immunoassay, in the serum of both BPH and prostate cancer patients comprising, on average, 25% of the fPSA fraction (Linton *et al.*, 2003). The Beckman Coulter automated BPSA immunoassay is named Access BPHA assay and it is available for research use only (Stephan *et al.*, 2009a).

The clinical value of BPSA seems to be much like that of fPSA-N. BPSA has been found to correlate with prostate volume (Canto *et al.*, 2004; Naya *et al.*, 2004). Thus, it may be more useful in discriminating BPH patients from prostate cancer patients than in a screening setting but the results from different studies are somewhat controversial (Jansen *et al.*, 2010; Khan *et al.*, 2004; Sokoll *et al.*, 2008; Stephan *et al.*, 2009a). Again, it is likely that this form of fPSA is not clinically useful alone but rather used in relation to tPSA, fPSA or [-2]proPSA or in combination with them in a statistical model (de Vries *et al.*, 2005; Stephan *et al.*, 2009a). The data regarding the ability of BPSA to predict aggressive cancers is also contradictory and no definitive conclusions can be made yet (de Vries *et al.*, 2005; Jansen *et al.*, 2010; Stephan *et al.*, 2009a).

2.2.7 Other free PSA forms

Glycovariants

The glycosylation patterns of proteins are different in cancer cells than in normal cells and this difference could be detected and used in diagnostics (Drake *et al.*, 2010). PSA has one N-linked glycosylation site at Asn45 (Belanger *et al.*, 1995) and different glycosylation patterns between normal, BPH and prostate cancer have been found (Li *et al.*, 2011; Ohyama *et al.*, 2004; Peracaula *et al.*, 2003; Tabares *et al.*, 2007; Tabares *et al.*, 2006; White *et al.*, 2009). It seems that in prostate cancer PSA forms contain less sialic acid and more fucose than BPH derived PSA (Dwek *et al.*, 2010; Sarrats *et al.*, 2010). The research on the clinical significance of different glycovariants of serum fPSA has been rather limited and it has been hindered by the technological obstacles due to the requirements for sensitivity (high PSA concentrations needed) or demanding technology which is not readily available (Dwek *et al.*, 2010). Recently, enzyme-linked lectin assays (ELLAs)

have been published (Dwek *et al.*, 2010; Meany *et al.*, 2009), which may ease further studies to clarify the clinical significance of different glycovariants.

Alternatively spliced variants

Several different alternatively spliced variants of *KLK3* gene have been found (David *et al.*, 2002; Heuze *et al.*, 1999; Heuze-Vourc'h *et al.*, 2003; Pampalakis *et al.*, 2008; Schulz *et al.*, 1988; Tanaka *et al.*, 2000). Many of them have been detected on mRNA level but only a few on protein level in prostate tissue or prostate cancer cell lines (David *et al.*, 2002; Heuze-Vourc'h *et al.*, 2003; Kumar *et al.*, 2000). To date, there are only speculations that these protein variants could be present also in blood circulation but whether they would have any clinical significance remains to be seen.

2.3 Kallikrein-related peptidase 2 (hK2) in prostate cancer diagnostics

2.3.1 Structure and function of hK2

Human kallikrein-related peptidase 2 (KLK2, hK2) shares 79% of its amino acid sequence with PSA (Schedlich *et al.*, 1987) as well as many epitopes which are recognized by antibodies developed originally against PSA (Piironen *et al.*, 1998). Like PSA, it is produced almost exclusively in the prostate and secreted into the prostatic fluid (approximately 0.1–2% of PSA concentrations). hK2 has trypsin-like substrate specificity and it is thought that hK2 activates proPSA in the semen but it may also cleave semenogelins. (Deperthes *et al.*, 1995; Deperthes *et al.*, 1996; Kumar *et al.*, 1997; Lovgren *et al.*, 1997; Magklara *et al.*, 2000; Takayama *et al.*, 1997). In seminal plasma hK2 forms a complex with PCI (SERPINA5) within ten minutes and this complex is the major form found in semen (Deperthes *et al.*, 1996; Grauer *et al.*, 1996; Heeb and Espana, 1998). In addition, hK2 has most probably an important role in activating other kallikreins and proteins in enzyme cascades (Lawrence *et al.*, 2010).

Unlike PSA, most of the serum hK2 is in free form but some of the hK2 forms complexes with ACT (Becker *et al.*, 2000a; Black *et al.*, 1999; Grauer *et al.*, 1998). The complex with PCI has not been found in serum (Grauer *et al.*, 1998) and a possible complex with A2M would not be detectable by conventional immunoassays. There is a lack of information about other complexed hK2 forms in serum. The free hK2 (fhK2) may occur in serum as a mature form as hK2 is able to autoactivate (Denmeade *et al.*, 2001; Mikolajczyk *et al.*, 1997b). It is also probable that nicked forms of fhK2 are present in the blood circulation (Lovgren *et al.*, 1999; Mikolajczyk *et al.*, 1999). Due to the close resemblance between hK2 and PSA it is likely that other fhK2 variants similar to fPSA variants exist. There may be variation in length of the peptide backbone and in glycosylation, splicing or nicking. Despite the majority of the circulating hK2 being in free form, the ratio of fhK2 to total hK2 (thK2) seems to vary between 15–100% (Vaisanen *et al.*, 2004; Vaisanen *et al.*, 2006).

In general, hK2 concentrations in blood are less than 2% of tPSA concentrations which requires very sensitive assays for detection of circulating hK2. As the covariation of PSA and hK2 in circulation is < 60% and their immunohistochemical expression patterns in prostate tissue are different, hK2 is considered to be independent of PSA as a potential marker (Charlesworth *et al.*, 1997; Piironen *et al.*, 1996a; Tremblay *et al.*, 1997).

2.3.2 Total and free hK2 in prostate cancer diagnostics

It has been found in several studies that hK2 concentrations are elevated in patients with prostate disorders. Whether measuring hK2 concentrations is of an independent clinical value has been a more controversial issue. There are studies that report hK2 measurement being helpful in discriminating prostate cancer patients from non-cancer patients and other studies where hK2 has been shown to add information in predicting the cancer stage or grade pre-operatively (Becker *et al.*, 2003; Haese *et al.*, 2001; Kwiatkowski *et al.*, 1998; Recker *et al.*, 1998). But there are also contradicting results for both cases (Bangma *et al.*, 2004; Kurek *et al.*, 2004; Stephan *et al.*, 2006a; Steuber *et al.*, 2007a). So far it seems that hK2 is most useful in combination with fPSA or other PSA forms. This has been supported by studies in screening settings which aimed at the prediction of cancer risk in biopsy (Becker *et al.*, 2000b; Nam *et al.*, 2000; Raaijmakers *et al.*, 2007; Vickers *et al.*, 2010a; Vickers *et al.*, 2008) and also in radical prostatectomy patients for predicting cancer aggressiveness and biochemical recurrence of the cancer (Haese *et al.*, 2001; Haese *et al.*, 2005; Steuber *et al.*, 2006; Steuber *et al.*, 2007b; Wenske *et al.*, 2009). The kallikrein panel including thK2 is discussed in more detail in chapter 2.5.1.

In these studies, often only thK2 has been measured. Both of the two most commonly used immunoassays are for research-use only (Becker *et al.*, 2000a; Blijenberg *et al.*, 2003; Finlay *et al.*, 2001; Piironen *et al.*, 1996a; Vaisanen *et al.*, 2004). There are differences in the assay constructs and standardization (discussed in chapter 2.4.2) which may partly explain the contradictory results. The intraindividual variation seems to be rather low (Christensson *et al.*, 2011) at least in the short term. There is a lack of information on different fhK2 forms and the ability of different assays to recognize them or thK2 in equimolar fashion. So far it seems that measuring fhK2 does not offer additional information on thK2 (Steuber *et al.*, 2007b; Vaisanen *et al.*, 2004; Vaisanen *et al.*, 2006) but the number of studies is so low that no final conclusion can be drawn yet. If new information regarding all possible fhK2 forms is discovered fhK2 may again become more interesting from a clinical point of view.

2.4 Challenges with immunoassays

2.4.1 Immunoassay interference

Analytical interference is an error in the measurement caused by the sample component which is not, by itself, producing a signal in the assay. Immunoassay results may be falsely high or low due to different factors. The sample handling (too early centrifugation, freezing and thawing) and storage (temperature, time) as well as the nature of the sample

(serum or anticoagulated plasma) may affect the measurement. The sample may contain fat, debris, clots or aggregates of fiber that interfere with the measurement (Selby, 1999).

Clinical impact of assay interference

It is important to recognize the possibility of false results especially when the clinical decision depends on the test result. There are unfortunate cases of misdiagnosis and mistreatment due to false immunoassay results (Cole *et al*., 1999; Ismail *et al*., 2002). The problem is pronounced for the very sensitive assays where even a slightest signal is significant.

In the normal clinical setting where men are tested for PSA level a slight increase or decrease in the PSA concentration is probably insignificant if the level stays below the commonly used thresholds of 3 or 4 ng/mL. If the false signal is markedly high it may lead to unnecessary biopsies but as the final decision on treatment is not based solely on the PSA value the chance of mistreatment will decrease. Only in the case where the prostate cancer patient has been treated by radical prostatectomy and the possible recurrence is followed by regular PSA measurements even an increase from 0.01 ng/mL to 1 ng/mL may cause unnecessary treatment. There are some reported cases where the PSA concentration was falsely elevated due to assay interference after a radical prostatectomy (Camacho *et al*., 2002; McAuley *et al*., 2002; Park *et al*., 2007) but the patient was unnecessarily treated for recurrent cancer only in a few cases (Fritz *et al*., 2009; Morgan and Tarter, 2001).

Antibody-binding components in blood

Different antibody binding components in the blood cause interference especially in two-site immunoassays (**Figure 3**). A falsely high signal occurs when the tracer antibody is bound to the capture antibody through the interfering factor when no antigen is bound to the antibodies. A falsely low signal occurs when either the capture or tracer antibody is incapable of binding the antigen because of the interfering factors. The most common interfering factors are either complement factors or different immunoglobulins produced by human immune system.

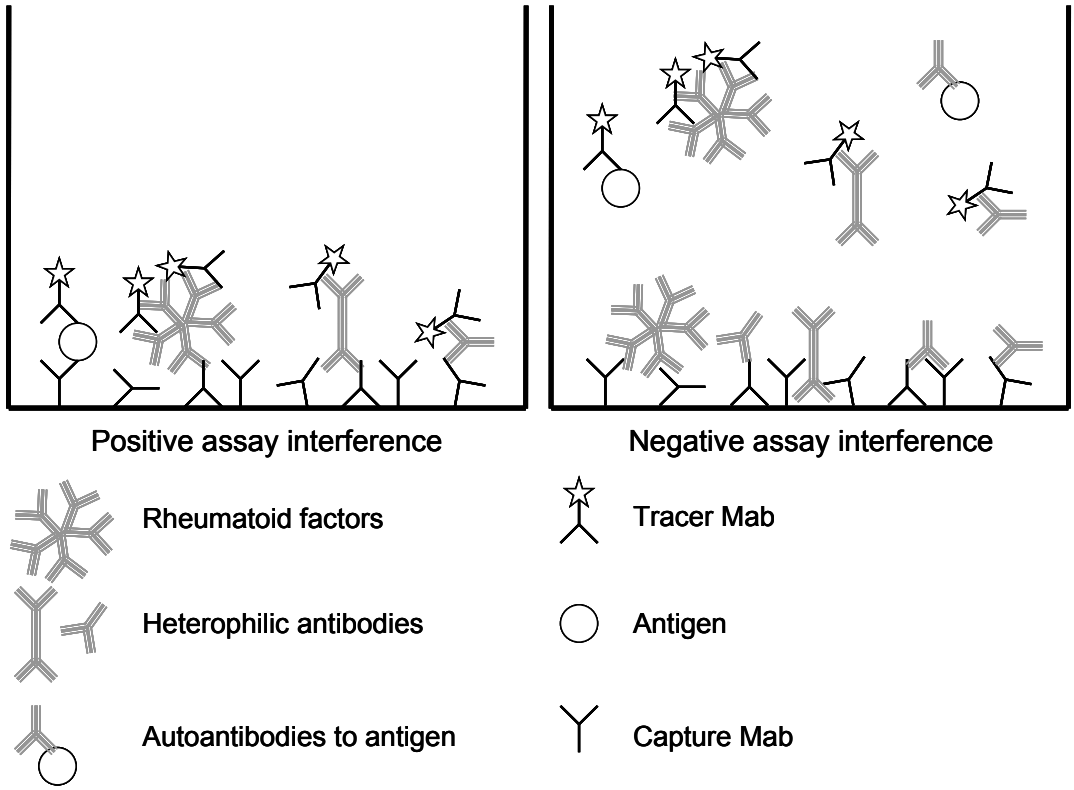

Figure 3. Immunoassay interference in two-site immunoassays. The desired signal from the complex of antigen and assay antibodies is shown on the far left. Positive assay interference bridges the labeled tracer antibodies unspecifically to the capture antibody. Negative assay interference prevents the formation of the desired complex by blocking the required binding sites of the antibodies.

Complement activation involves binding of the complement proteins to the Fc-part of aggregated IgG molecules. It is natural that in immunoassays where a solid phase is occupied by stacked IgG molecules the complement may attach to the surface and either block the binding of the antigen by steric hindrance and cause a falsely low signal or also the tracer antibody forming a bridge resulting in a falsely high signal. (Baatrup *et al.*, 1986; Kapyaho *et al.*, 1989)

Rheumatoid factors are human autoantibodies of any class but mainly IgM which recognize the Fc part of human immunoglobulins. They may also recognize immunoglobulins from other species and thus cause interference in the immunoassays. The rheumatoid factors are present in approximately 70% of rheumatoid arthritis patients but they are also present in patients with other autoimmune diseases and in about 5% of normal population (Courtenay-Luck *et al.*, 1987; Wolfe *et al.*, 1991).

Heterophilic antibodies are human antibodies of any subclass which bind to any part of murine (or other animal) antibody, are of sufficient titer and affinity to have an

analytically significant effect and there is no known immunogen (Bjerner, 2005; Kaplan and Levinson, 1999). They are differentiated from the specific antibodies against animal antibodies (human anti-animal antibodies, HAAA) which are caused by for example therapeutic or imaging use of animal antibodies (the immunogen is known) or sometimes when the patient has a history of handling animals. The *in vivo* use of mouse antibodies for treatment or diagnostics is common and will cause the immune system to produce human anti-mouse antibodies (HAMAs) which may be very specific for the antibody used but may sometimes be of less specificity and interfere with the immunoassays even though the administered antibody is not used in the assay. (Kaplan and Levinson, 1999; Kricka, 1999).

The heterophilic antibodies are mainly of IgM class and they may be specific to murine IgG subclass (Bjerner *et al.*, 2005). Thus they are prone to bind to the tightly stacked mouse monoclonal IgGs bound on the solid surface of the assay. They, as well as the specific HAMAs, often bind to the Fc part of the antibody (Bjerner *et al.*, 2002; Thorpe *et al.*, 2003).

The frequency estimations of the heterophilic antibodies and HAAAs differ depending on the population and assay method used. The reported frequencies vary from 3.4% to 52% (Bjerner *et al.*, 2002; Boscato and Stuart, 1986; Frengen *et al.*, 1994; Hawkins *et al.*, 1980; Koshida *et al.*, 2010; Ward *et al.*, 1997) and even a prevalence of 0.53% has been reported (Ismail *et al.*, 2002) but the latter study was conducted with commercial assays in which the interference may have been reduced by blockers in the assay reagents.

Autoantibodies against the antigen may cause falsely low signals by inhibiting the binding of the antigen to the assay antibody. This problem has been found for example in immunoassays measuring a cardiac marker troponin I (Eriksson *et al.*, 2005). There are also reports of autoantibodies towards PSA both in BPH (Zisman *et al.*, 1995) and prostate cancer patients (McNeel *et al.*, 2000) but their impact on the measured PSA levels remains uncertain.

Removing interference

The most common way to reduce the assay interference is to use different blocker agents in the assay buffer. Most unwanted protein-protein interactions are avoided by adding bovine serum albumin (BSA) and detergents, such as polysorbates.

The complement is a problem mainly for fresh serum samples and complement activity progressively decreases when the samples are stored at +4 °C or when the samples are diluted. Also, as Ca^{2+} ions are needed to activate the complement the use of EDTA plasma prevents the interference caused by the complement. (Kapyaho *et al.*, 1989; Weber *et al.*, 1990).

Adding an excess of different immunoglobulins, like bovine gammaglobulin, into the assay buffer reduces the interactions between immunoglobulins and proteins binding to them (**Figure 4**). Assays with mouse antibodies usually benefit from an addition of either mouse serum or purified unspecific mouse immunoglobulins (often IgG). These scavenger

antibodies should prevent the binding of the heterophilic and other antibodies in the sample to the assay antibodies. Specific commercial blockers are available, for example MAK-33 (Roche Molecular Biochemicals) and HBR (Heterophilic Blocking Reagent, Scantibodies) of which MAK-33 is an irrelevant mouse monoclonal IgG and HBR a mouse monoclonal IgG against human IgM. Denaturation or polymerization of the blocking agents have been found to enhance blocking efficiency (Bjerner *et al.*, 2002; Lenz *et al.*, 1990).

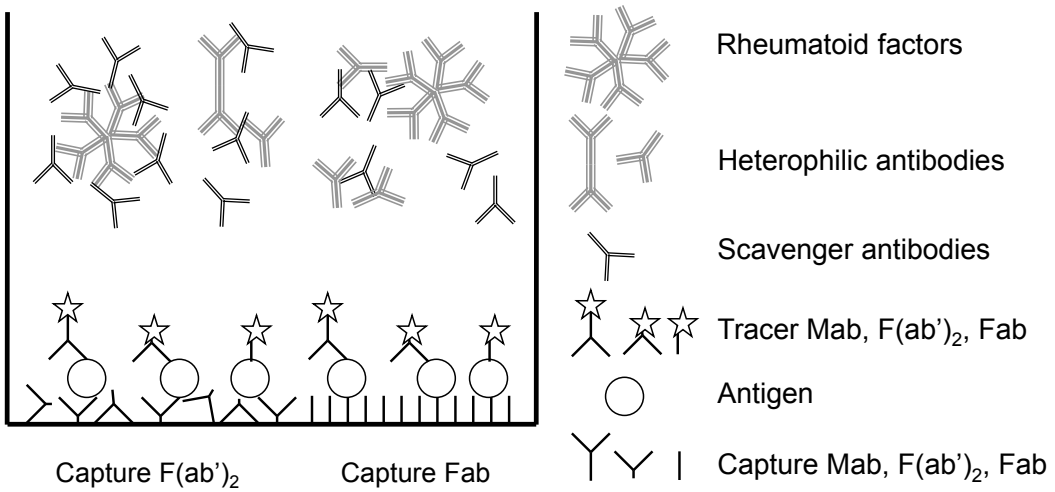

Figure 4. Removing immunoassay interference. Scavenger antibodies can be used to remove assay interference by having an excess of them together with the sample. The interfering factors are expected to bind the scavenger antibodies instead of the capture and tracer antibodies. The interfering factors often recognize the Fc part of the antibody and thus, the use of capture or tracer antibodies as antibody fragments without the Fc part ($F(ab')_2$ or Fab fragments) reduces the assay interference.

Redesigning the assay may help in reducing interference problems. Selecting antibodies of different animal origin or subclass has been shown to have some effects (Hennig *et al.*, 2000; Larsson *et al.*, 1992) as has the use of chimeric antibodies (Kuroki *et al.*, 1995). The most efficient way to reformulate an assay to reduce the interference is to use antibody fragments lacking the Fc part because it is the portion to which the complement proteins and most of the heterophilic antibodies and rheumatoid factors most often bind. Enzymatically produced or recombinant Fab or $F(ab')_2$ fragments as well as recombinant scFv fragments have, in several studies, been shown to be effective even though this will not eliminate the interference completely (Bjerner *et al.*, 2002; Csako *et al.*, 1988; Vaidya and Beatty, 1992; Warren *et al.*, 2005). The most efficient removal of interference is most likely accomplished by combining the use of antibody fragments and at least some immunoglobulin blocker in the buffer.

The HAMAs (and other HAAAs) may be very specific for the particular antibody which has been administered to the patients and these administered antibodies should not be used

in assay constructs. After repeated administrations the HAMAs may develop a specificity against the antigen-binding site (idiotype) of the administered antibody and as a consequence they may start to compete with the actual antigen for the binding to the assay antibodies (Frodin *et al.*, 1992; Reinsberg and Nocke, 1993). This causes negative interference which may be very difficult to detect. The situation is similar when there are autoantibodies against the antigen. If this is suspected the presence of the anti-idiotypic or autoantibodies may be verified by recovery tests with known amounts of the antigen added to the sample. If the assay result is significantly different from the expected value some kind of negative interference may be present. The only way to achieve the correct value is to remove all immunoglobulins from the sample by, e.g., chromatography (Turpeinen *et al.*, 1990).

2.4.2 Standardization and different assay constructs

PSA

Many antibodies recognizing PSA were originally raised against fPSA purified from seminal plasma and, therefore, they may prefer binding to fPSA instead of PSA-ACT. This non-equimolar recognition becomes a problem because samples from patients with BPH or other benign conditions have more fPSA and thus the tPSA concentration may appear to be higher than the actual value (Semjonow *et al.*, 1996). Similar problems may occur in assays in which the binding reactions of antigen to antibody are not allowed to reach equilibrium state and if the rate of binding is different for fPSA and PSA-ACT (McCormack *et al.*, 1995). The problem of non-equimolarity was one of the main reasons different assays to give different tPSA concentrations even for the same sample (Graves, 1993a; Graves, 1993b). To address this problem 83 PSA antibodies were characterized in an antibody epitope mapping workshop which was organized by the International Society of Oncology and BioMarkers (ISOBM) in the late 1990's (Stenman *et al.*, 1999).

A further problem, differential standardization of assays, was another major cause for different results and in 2000 WHO launched the First International Standard for PSA (free) and also for PSA (90:10). Both originate from seminal plasma and the latter is a mixture of PSA-ACT (90%) and fPSA (10%). (Rafferty *et al.*, 2000)

The use of the WHO calibration has diminished the variation between different assays but still the concentrations are not fully interchangeable preventing, for example, the adoption of uniform recommendations for tPSA (or F/T PSA) thresholds for biopsy indication (Kort *et al.*, 2006; Roddam *et al.*, 2006; Stephan *et al.*, 2006b). The reason for this discrepancy, apart from calibration, is most likely in the different assay constructs – different antibodies and procedures (Semjonow *et al.*, 2000). For example, antibodies may have different affinities and in the procedures the incubation times may vary.

As stated, there may be clinical consequences of the methodological differences. For example, the Beckman Coulter original Hybritech Tandem-R calibration has been widely used and now the company is offering both the original and the WHO calibration for their PSA assays. By using the WHO calibration the results are approximately 20 to 25% lower than using the conventional Hybritech calibration (Fillee *et al.*, 2010; Jansen *et al.*, 2008;

Stephan *et al.*, 2008). This would also have clinical implications if the currently used threshold for further examinations is not adjusted accordingly.

There are many statistical models which are used to predict cancer risk before the initial biopsy and Stephan *et al.* studied whether different risk nomograms are predictive if the tPSA or fPSA concentrations are measured with an assay different from the one used for creating and validating the model (Stephan *et al.*, 2011). The differences were large and could lead to unacceptable variation in prostate cancer risk estimations. Especially the fPSA measurements seem to be prone to variation, whether calibrated according to WHO calibration or not, (Kort *et al.*, 2006; Semjonow *et al.*, 2000; Stephan *et al.*, 2006b; Stephan *et al.*, 2011) and thus the calculation of F/T PSA should be made from previously studied combinations of assays to draw any conclusions. The fPSA variation in measurements may be caused by the differential recognition of the various fPSA forms found in blood. In general, each assay should have its own reference ranges and when a statistical model is used the assay should be the same that was used for the development and validation of the model. The clinical laboratory should inform clinicians if the assay method used by the laboratory is changed and provide the PSA concentrations from both assays for a while to familiarize the clinician with the new method.

hK2

There is no international standard material to calibrate hK2 assays. Recombinant ek-rhK2 which has mutated pro-sequence to prevent autoactivation (Lovgren *et al.*, 1999), was recommended as a potential standard material due to its ability to improve the agreement between the Hybritech (Beckman Coulter) thK2 assay and time-resolved fluorometric assay for thK2 developed by research groups in Scandinavia (Haese *et al.*, 2003c). There are significant differences between the two assays even after common calibration and the results are, most likely, not interchangeable. The reason for the differences between the assays may be in the possible different hK2 forms in the blood, assay constructs or other, for example sample related issues.

2.4.3 Stability of PSA and hK2 in sample material

If handled and stored appropriately, total PSA is rather stabile in both serum and anticoagulated plasma, even for several years but fPSA seems to be more vulnerable to degradation especially in serum (Pettersson *et al.*, 1995; Piironen *et al.*, 1996b; Ulmert *et al.*, 2006; Woodrum *et al.*, 1996). Of the different fPSA forms the stability of fPSA-I and [-2]proPSA have been studied and they seem to resemble fPSA in their stability characteristics, tending to degrade faster in serum. Therefore, either fast measurement, freezing or collection of anticoaculated plasma is recommended to retain the protein concentration (Nurmikko *et al.*, 2001; Semjonow *et al.*, 2010).

There is little published information regarding the long-term stability of hK2. The stability study made with the Hybritech (Beckman Coulter) hK2 assay suggests that hK2 is rather stabile in serum but should be measured soon after blood draw or, for long term storage, should be kept at –70 °C (Finlay *et al.*, 2001). For the time-resolved fluorometric assay (Vaisanen *et al.*, 2004) the short term stability seemed to be generally good both at +4 °C

(over three weeks) and at room temperature (up to 120 h) in either serum or heparin, EDTA or citrate plasma. However, hK2 concentration in individual samples could fluctuate even up to 2.5 fold during the storage at room temperature (V. Väisänen, unpublished results).

2.5 Diagnostic tools for prostate cancer

2.5.1 Statistical models

Regression models and artificial neural networks

Linear and logistic regression models as well as artificial neural networks (ANNs) are some of the most common statistical models used in the studies regarding the prostate cancer diagnostics. Linear regression is used not only for calculating standard curves but also for estimating whether some other factors (variables) may affect the linear relationship of variables x and y. For example, it is known that PSA values increase with age and it is possible to fit a linear function to a data set consisting of the PSA concentration and age of a group of men ($y = a + bx$, where y is PSA concentration, x is age, a is constant and b is coefficient). It is possible to study whether prostate volume has a relationship with PSA concentration when the effect of age is taken into account. This is done by adding another variable b_2x_2 to the equation where b_2 is the regression coefficient implicating the value or effect of the new variable x_2 (prostate volume) in the model. There may be several variables and they can be chosen so that only (clinically relevant) statistically significant variables are left in the final model. Because linear regression is used for studying the association of, for example, biomarkers and linear parameters of the disease, it is not usually used to classify individuals into different groups (with or without cancer risk etc.). The variables' value in discrimination of groups of individuals may be further studied by other methods like logistic regression or ANNs.

Logistic regression is used to predict a probability of an event which may or may not happen, i.e. it has two values (0 or 1). For example, a patient either has a cancer or he does not or, the cancer is either organ-confined or non-organ-confined. The function fitted to the data is a logistic function $f(z) = e^z / (1+ e^z)$ where variable z is consisting of i number of risk factors b_ix_i (plus the intercept term b_0). Again, b_i is the regression coefficient implicating the effect of each variable in the model. Similarly to linear regression, the final model can be chosen to include only statistically (and clinically) significant variables.

ANNs are more complicated functions which have more parameters than logistic regression. The possible interaction of variables (i.e. variables are affecting each other) and nonlinear relationships are taken into account. The ANN is trained with a set of data to find (complex) patterns in the data, and after this learning phase, it can be applied to another set of data to predict, for example, the risk of cancer. There is no possibility to evaluate the significance of a single variable in the ANN model and in that sense the ANN is considered a "black box" (Cammann *et al.*, 2011).

Model characteristics

There is a risk of over-fitting which means that the model may predict the outcome perfectly in the training set of data but when it is applied to another set the it does not predict the outcome anymore. For this reason there should be a validation data set where the outcome is known as in the training set but which consists of new observations. In this way the model functionality can be checked independently. Usually the original data set is divided into training and validation sets, which means that the number of observations needed is rather high. Even if the model has been internally validated in this manner it may not be directly applicable to a totally new data set, such as another population. For example, the model may have been developed based on European patients but it is not self-evident that it will work correctly when used for North American patients due to differences in populations. For using the model in another population it has to be validated externally for the population in question.

The quality of a prediction model can be estimated by how good the model is in discriminating the two groups (healthy and cancer patients) and how well the model is calibrated meaning the extent to which the probabilities predicted by the model actually agree with the observed probabilities (or outcomes). Common terms to describe diagnostic methods are explained in the **Table 4**.

Table 4. The terms used to describe a diagnostic method.

		Condition ("truth")		
		no cancer	**cancer**	
Test result	**no cancer**	true negatives	false negatives	**Negative predictive value:** $\frac{\text{true negatives}}{\text{true negatives + false negatives}}$
	cancer	false positives	true positives	**Positive predictive value:** $\frac{\text{true positives}}{\text{false positives + true positives}}$
		Specificity: $\frac{\text{true negatives}}{\text{true negatives + false positives}}$	**Sensitivity:** $\frac{\text{true positives}}{\text{false negatives + true positives}}$	

The model's ability to discriminate two groups is commonly visualized with a receiver-operating characteristic (ROC) curve where sensitivity is plotted against false positive rate (1 – specificity) and expressed as the area under the ROC curve (AUC, see **Figure 5**). The AUC of 0.5 means that the model does not discriminate the two groups (the same as a flip of a coin). The higher the AUC above 0.5, the better the discriminatory power of the models are. The shape of the curve may be different for two models even though the AUCs are the same meaning that the models' ability to separate the groups at a given sensitivity may be different. This makes the comparison of the models somewhat more difficult. Also other relating numbers, for example, positive and negative predictive values (PPV and NPV, see **Table 4**.) and specificities at certain sensitivity levels can be used to describe the model performance. The calibration can be evaluated, for example, by quantile-quantile plots where the distributions of the predicted and observed probabilities are plotted. If the distributions are similar the points align on a diagonal (45°) line. If the points do not lie on this line, the position of those points show whether the model under-

or overestimates the prostate cancer risk, and whether this is general or just in certain areas.

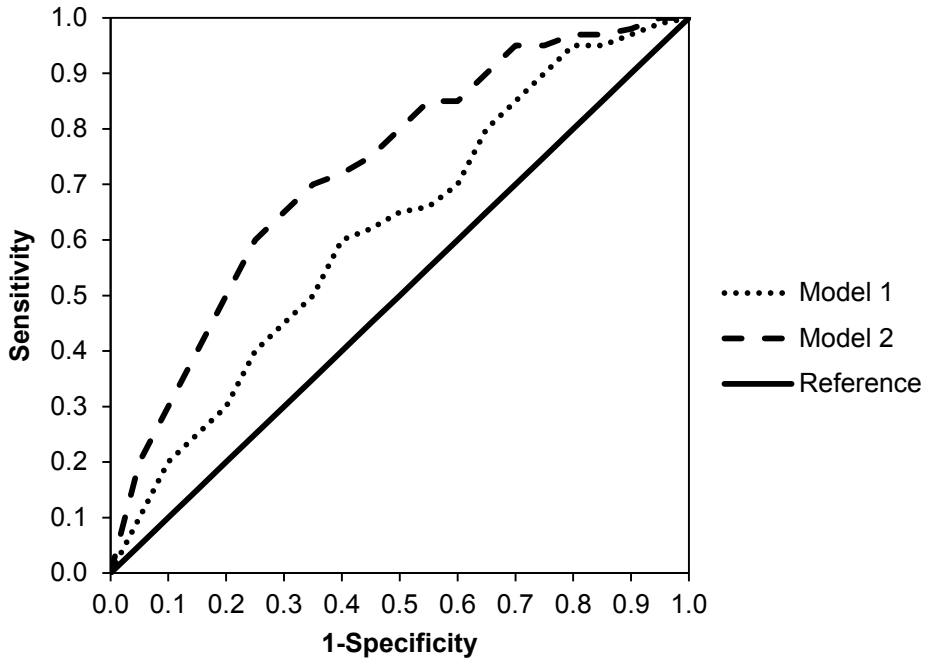

Figure 5. Receiver-operating characteristic (ROC) curve and AUC. The ROC curve illustrates the discrimination of two groups (patients with and without cancer) by parameters, which are in this example the Model 1 (dotted line) and Model 2 (dashed line). The reference line (solid line) shows the situation where there is no discrimination. The area under the ROC curve (AUC) is in this case 0.5. Of the models, the Model 2 can discriminate the two groups better as the line is closer to perfect discrimination (the upper left corner) than the line of Model 1. The AUC of Model 2 is higher than that of Model 1 (exact AUCs have not been calculated).

Comparing models

Whether one model discriminates the groups better than the other can be evaluated by comparing the AUCs of the two models on same data (or on different data if the models are being validated externally). The model with the larger AUC discriminates the groups better statistically (see **Figure 5**). This does not necessarily mean that the other model is clinically more useful.

The clinical value can be evaluated by a decision curve analysis (Vickers and Elkin, 2006) which takes into account the fact that each decision will have consequences which may be beneficial or harmful and that individuals may weigh those consequences differently. A common example of using decision curve analysis is a situation where, after PSA measurement (and maybe also some clinical workup), a patient and a clinician consider whether the patient has a clinically significant prostate cancer risk and a biopsy should be

taken. As biopsy may have some negative consequences some men might want the biopsy taken if the cancer risk lies at or above 30% (threshold probability) while some may want to be biopsied if the risk lies at 10% or above, i.e. the men weigh the relative harm of a false-positive and false-negative predictions differently. The net benefit for each threshold probability is estimated by adding up the benefits (true positives) and subtracting the harms (false-positives). The latter is weighed by a factor which is derived from the threshold probability. In the decision curve (**Figure 6**) the net benefit is plotted on the y-axis and the threshold probability (estimated cancer risk by the model) is plotted on the x-axis. The curves of different models, including options of treating (biopsying) all or none, are drawn and the model having the highest net benefit at certain (clinically relevant) threshold probability or cancer risk (range) is the one that should be used.

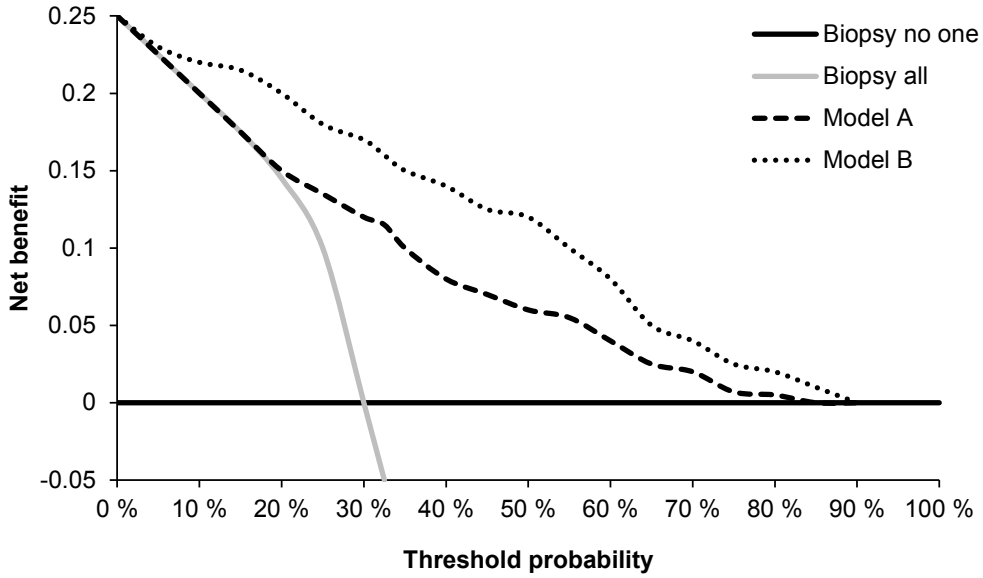

Figure 6. Decision curve analysis. The relative harms and benefits of the decision (biopsy or not) are expressed as net benefit and the threshold probability (probability to have cancer at biopsy) at which one might choose either alternative are plotted on the y- and x-axis respectively. In this example, should a man choose biopsy when the prostate cancer risk is 20% or higher, Model B would give the best net benefit while Model A would give the same result as biopsying all men.

Models for predicting prostate cancer risk

The models including PSA measurements in them (most of them are) seem to be tied to the PSA assay method (in addition to the population cohort) used while developing them. This was noted in a recent study where the predicted probabilities varied significantly if PSA values form other assays were applied (Stephan *et al.*, 2011). This is an important issue to recognize when the models are utilized in clinical practice.

Kallikrein panel consists of measured concentrations of tPSA, fPSA, fPSA-I and thK2. A laboratory base model including age and tPSA and a clinical model including DRE in addition to the subject's age and tPSA were developed using multivariable logistic regression and compared with similar models with the additional three kallikrein forms (Vickers *et al.*, 2008). The models were developed with the ERSPC screening cohort from Gothenburg, Sweden, comprising 740 men undergoing biopsy within the first screening round due to elevated tPSA of ≥ 3 ng/mL in order to study the kallikrein panel's ability to help in predicting the presence of cancer in the prostate biopsy (192 men had cancer). The result was that using this kallikrein panel a significant number of unnecessary biopsies could have been avoided. Using a 20% probability of prostate cancer as the threshold, 57% or 60% of the biopsies could have been avoided using the laboratory or clinical models with kallikreins, respectively.

A low number of cancer cases were missed (31 or 33 of 152 low-grade cases of cancer with laboratory and clinical models, respectively) and only few of them were high-grade cancers (3 or 1 of 40 high-grade cancer cases). In the decision curve analysis these full models including all kallikreins had better net benefit than the base models or base models with fPSA over the studied range of threshold probabilities (5–75%). These results were later repeated and thus externally validated with another ERSPC cohort form Rotterdam, The Netherlands (Vickers *et al.*, 2010a).

Additionally, the kallikrein panel also predicts the biopsy outcome in recently screened men (Vickers *et al.*, 2010b; Vickers *et al.*, 2010c) with elevated tPSA – a subgroup in which tPSA (or the base model including age) loses its ability to predict the biopsy outcome due to the tPSA based screening. The kallikrein panel has also been applied to men with previous negative biopsy (Gupta *et al.*, 2010) and pre-biopsy work-up (Benchikh *et al.*, 2010) and again, the number of unnecessary biopsies could be significantly reduced with only a few, mostly low-grade cases of cancer missed. To study whether the kallikrein panel would truly help in reducing the over-diagnosis of early-stage and low-grade cancers the panel was applied to men who attended the Malmö Diet and Cancer study (Manjer *et al.*, 2001) during 1991–1996 by providing a blood sample (Vickers *et al.*, 2010e). Their cancer status at the end of year 2005 was used to calculate the number of men with tPSA ≥ 3 ng/mL, who would have been advised against biopsy if kallikrein panel had been used but who would have developed a clinically diagnosed cancer within 5 years after the blood draw. There would have been 21 in 1000 men with clinical cancer and only two of them would have been diagnosed with advanced cancer. Therefore, the use of the kallikrein panel could also reduce over-diagnosis and the model would reduce the number of biopsies by 42%. (Vickers *et al.*, 2010e.)

As it is notable that the kallikrein panel was developed with screening cohorts and with specific immunoassays there is no data on how the panel would work with referral patients (even though they might resemble the cohort with previous clinical work-up) or with PSA and hK2 concentrations measured with assays from other manufacturers. To date the fPSA-I and hK2 assays used in the kallikrein panel are not commercially available unlike the models discussed in the following chapters.

Prostate health index (phi) is a mathematical function developed by Beckman Coulter. It consists of measurement of tPSA, fPSA and [-2]proPSA (with p2PSA assay) and the concentrations are combined into a formula: ([-2]proPSA/fPSA) x √tPSA. The phi was launched in 2009 and to date only a few studies investigating the clinical significance of phi have been published. Generally, the published studies suggest that phi could help in discriminating prostate cancer patients from men without cancer (Catalona *et al.*, 2011; Guazzoni *et al.*, 2011; Jansen *et al.*, 2010; Le *et al.*, 2010). The increasing phi seems to be associated with increasing cancer risk and the AUCs for predicting prostate cancer in initial biopsy have been between 0.70 and 0.77, which indicated that phi discriminated patients better than using only tPSA or F/T PSA. At sensitivities around 90% the specificity of phi was 20–35% (in comparison to specificities of tPSA < 10% and F/T PSA 10–23%). The phi is to be used only with Beckman Coulter PSA assays and there are separate reporting criteria for the Hybritech and WHO calibrated assays. The company provides three-class risk classification tables on their web site to help clinicians to interpret the phi result (Beckman Coulter, 2012).

There are also several other prediction models but few of them have been validated externally (Schroder and Kattan, 2008; Shariat *et al.*, 2008) and even fewer have gained popularity among urologists. The two of the most commonly used models are presented in the following chapter because there are user friendly Internet based prediction tools available on them.

2.5.2 From a model to a practical tool

Nomograms

A nomogram is a graphical presentation of a statistical model which is made to help the use of the model in practice (see **Figure 7**). Usually there are several segmented specifically aligned lines indicating the values which each variable may have, and a line where the points corresponding to those values can be seen by drawing a straight line from the variable value to the points. Another line indicates the total number of points which is aligned with a line where the final probability can be estimated again by drawing a straight line from the total points to the corresponding probability. It is possible to make the nomogram in different formats, for example as a handheld round disk where each layer correspond one variable. The layers are aligned by turning the disks according to instructions to obtain the probability of the disease, or prostate cancer as was done with ERSPC risk calculator (Kranse *et al.*, 2008).

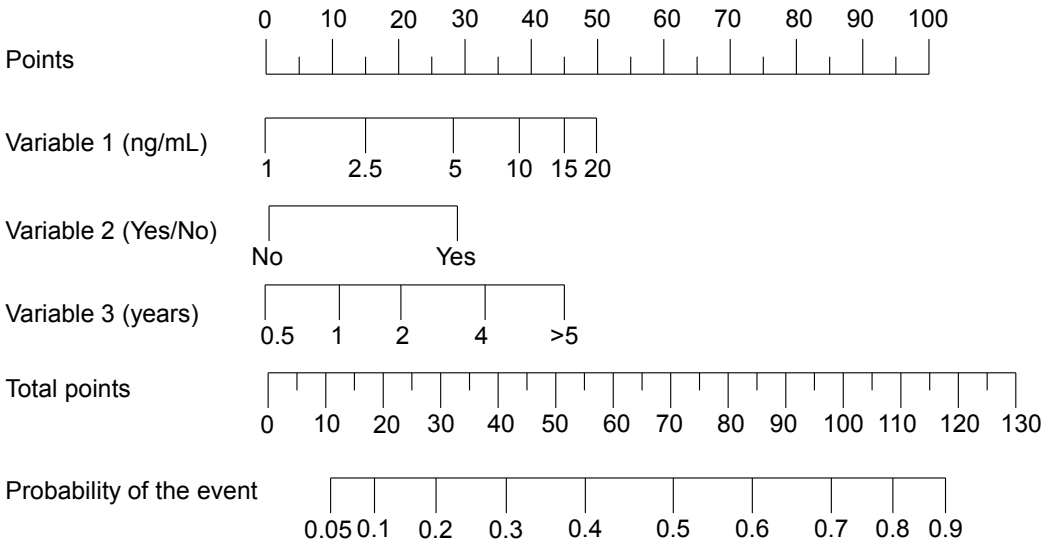

Figure 7. A generic example of a nomogram. The points of each variable are read from the line at the top. All points are added up and marked on the "Total points" line. The probability of the event is read from the bottom line at the position directly below the sum total.

The nomograms are usually tied to the characteristics of the population they were developed for and also to the variables they include. If a variable is not available it is often impossible to calculate the probability and another nomogram without that particular variable is needed. This may not be practical as there may be many variables which are of different value to different kind of populations.

The nomogram can also be applied as an Internet based calculator tool as was done with the above mentioned ERSPC risk calculator as well as the prediction model based on the Prostate Cancer Prevention Trial (PCPT). These risk calculators are discussed below.

Risk calculators

ERSPC risk calculators are nomograms of six logistic regression models based on the screening cohorts in ERSPC section Rotterdam. The risk calculators provide estimates of current prostate cancer risk based on age, family history and urinary complaints in calculator 1, tPSA only in calculator 2 and tPSA in combination with DRE, TRUS, prostate volume and previous biopsy status in calculators 3 for previously unscreened men, 4 for previously screened but not biopsied men and 5 for previously screened and biopsied men. The last calculator is intended for the calculation of the probability for having a potentially indolent prostate cancer and for aiding in choosing a treatment. (Kranse *et al.*, 2008; Roobol *et al.*, 2012; Steyerberg *et al.*, 2007) The risk calculators have been validated externally (Cavadas *et al.*, 2010; Roobol *et al.*, 2009a; Trottier *et al.*, 2011; van Vugt *et al.*, 2011) and have been shown to help in estimating the risk for an individual patient as well as in the decision-making to perform biopsy. Recently it was

suggested that the prostate volume estimation, which is made with TRUS for the risk calculator, and thus requires equipment and skills to perform, could be replaced by a DRE based volume estimation to further ease the use of the calculators (Roobol *et al.*, 2012).

The calculators are available at www.prostatecancer-riskcalculator.com (accessed 1.3.2012) where the user fills in the required information on a form after which the program reports the estimated risk. They are developed based on Beckman Coulter tPSA assay with Hybritech calibration as this was chosen to be the common screening assay in ERSPC (Schroder *et al.*, 2009). The sextant biopsy scheme used is also noteworthy, as this scheme may not be used anymore and 10- or 12-core schemes may be more common in contemporary clinical practice. This might cause the underestimation of the prostate cancer risk (Cavadas *et al.*, 2010).

PCPT risk calculator was based on data of 5519 men who participated in the prevention study in its placebo arm (Thompson *et al.*, 2006). The men were primarily healthy and their tPSA was below 3 ng/mL. The variables included in the calculator were age (≥ 50 years), family history, race or ethnicity, status of the possible prior biopsy, DRE finding and tPSA concentration (the latter two variables should be no older than 1 year). The calculator gives estimates of risks of prostate cancer and high-grade prostate cancer in prostate biopsy. The calculator is available at http://deb.uthscsa.edu/URORiskCalc/Pages/uroriskcalc.jsp (accessed 2.3.2012).

The calculator has been externally validated several times with contradicting results and it seems to be generally overestimating the cancer risk if the population to which the model is applied differs from the original population with which the model was developed (Ankerst *et al.*, 2012). When the PCPT and ERSPC calculators have been compared head-to-head, the ERSPC calculator has performed better than the PCPT calculator (Cavadas *et al.*, 2010; Trottier *et al.*, 2011). The differences in study populations may explain these results.

2.5.3 Future aspects

The future of prostate cancer diagnostics lies most probably in multiple markers combined together within a statistical model. Whether the markers are the ones in the kallikrein panel, some newer markers like prostate cancer antigen 3 (PCA3) (Auprich *et al.*, 2011), or some combination of protein and genetic markers (Aly *et al.*, 2011) together with clinical information remains to be seen. The future of PSA in all of its forms does not seem to be threatened and it is likely that it will be measured also in the future. There is certainly need for development in the statistical models for them to be truly reliable tools for the clinician. The methods for developing a flexible and adaptive risk estimation tool may already exist, perhaps in service of a totally different purpose (Vickers *et al.*, 2010d), and the innovative adaptation of those methods to diagnostics is just waiting to be discovered.

3 AIMS OF THE STUDY

The overall aim of the study was to optimize the immunoassays for fPSA-I, fhK2 and thK2 and use those assays to measure the marker concentrations in clinical samples in order to study the concentration levels in different cohorts of prostate cancer patients and without prostate cancer. Also, a statistical model using the markers was applied to study the association of the markers with prostate volume. Finally, a direct immunoassay for measuring fPSA-N was developed to improve the analytical performance over the calculation method used in previous publications.

The more detailed aims in the original publications were:

I To assess whether the assay interference observed in fPSA-I, fhK2 and thK2 assays could be eliminated by removal of the Fc portion from the capture or tracer antibodies.

II To measure the concentration level of different PSA forms (tPSA, fPSA, fPSA-I and calculated fPSA-N) in prostate cancer patients selected for radical prostatectomy and study the association of the markers and the pathological stage and grade of the cancer.

III To study the association of four kallikrein markers (tPSA, fPSA, fPSA-I and thK2) with prostate volume by applying a statistical model, based on the four kallikreins, which predicts the prostate biopsy outcome and to assess whether any of the markers or combination thereof could replace the volume measurement in predicting the biopsy outcome.

IV To develop a direct immunoassay for fPSA-N concentration measurements and to evaluate its analytical performance.

4 SUMMARY OF MATERIALS AND METHODS

The details of the materials and methods used are described in the original publications (**I-IV**).

4.1 Clinical samples

The summary of the different sample panels is described in the **Table 5**. All samples were obtained following standard hospital protocols and ethical procedures accepted by the ethical review board of each institution. The following study procedures were in accordance with the Helsinki Declaration of 1975, as revised in 1996. As the sample processing and storage conditions may affect especially the fPSA concentrations (see chapter 2.4.3), these procedures have been described in detail.

The routine heparin plasma samples collected at Malmö University Hospital (Malmö, Sweden) were stored at room temperature for up to 6 h and then transferred to +4 °C for 1–4 day storage before freezing at –20 °C. All identifying information was removed prior to sending the samples to the Department of Biotechnology, University of Turku and no clinical information except for gender was attached to the samples. fPSA, fPSA-I, thK2 and fhK2 concentrations of the samples were measured to find the samples with potential assay interfering factors (**I**). The selected samples were used to further study the interference problem. The samples used for studying interference fPSA-N (**unpublished)** were similar to other routine samples except that the immunoassay measurements were performed at Malmö University Hospital before the pooling at the Department of Biotechnology.

The routine serum samples collected at Turku University Hospital were without any identifying information and were collected identically to the routine samples at Malmö University Hospital. These samples were used to evaluate the optimized immunoassay protocols (**I**).

The clinically defined anti-coagulated plasma samples were collected from consecutive men that underwent clinical work-up due to their symptomatic prostate condition at the Department of Urology at Turku University Hospital. The samples were taken before biopsy or initiation of treatment, centrifuged within 3 h of the blood draw and stored at –20 °C. The samples were used to evaluate the optimized immunoassays (**I**), to study the pre-operative fPSA forms concentrations in radical prostatectomy patients (**II**) and to evaluate the fPSA-N immunoassay (**IV**).

The samples collected for the European Randomized Study of Screening for Prostate Cancer (ERSPC) were processed within 3 h of the venipuncture at the Rotterdam University Hospital, Rotterdam, The Netherlands and at the Sahlgrenska University Hospital, Göterborg, Sweden where they were stored at –80 °C and –20 °C, respectively. The samples were shipped in a frozen state to Malmö University Hospital where the kallikrein measurements were performed (**III**).

The plasma samples collected from healthy volunteers at the Department of Biotechnology were centrifuged within 1 h of the venipuncture and stored at –20 °C. The samples were used for fPSA-N immunoassay evaluation (**IV**).

Table 5. Description of the samples used in the studies. Samples obtained from one individual may have been used in more than one study.

Collection place	Sample type	Matrix	Gender	Number of samples	Publication and use
Malmö University Hospital	routine	heparin plasma	male female	957 1092	**I** assay interference studies
			female	31 (8)	**IV** fPSA-N assay interference study, (recovery study)
Turku University Hospital	routine	serum	male	171	**I** assay interference studies
	clinically defined patients	heparin plasma	male	138	**I** assay interference studies
				111	**II** fPSA forms in rp[a] patients
				13 (5)	**IV** fPSA-N assay evaluation (recovery study)
		EDTA plasma	male	111	**I** assay interference studies
				198	**II** fPSA forms in rp[a] patients
				63	**IV** fPSA-N assay evaluation
Rotterdam University Hospital	screening study population	serum	male	2914	**III** kallikreins and prostate volume
Sahlgrenska University Hospital, Göteborg	screening study population	serum	male	740	**III** kallikreins and prostate volume
UTU/BT[b]	healthy volunteers	EDTA plasma	male female	9 12	**IV** fPSA-N assay evaluation

[a] rp, radical prostatectomy
[b] UTU/BT, University of Turku, Department of Biotechnology

4.2 Antibodies and standard materials

4.2.1 Monoclonal antibodies (Mabs)

The monoclonal antibodies used are listed in the **Table 6**.

Table 6. The monoclonal antibodies (Mab) used in the studies.

Mab	Specificity	Immunoassay	Publication	Reference
11B6	fhK2	fhK2 capture	I-III	(Vaisanen *et al*., 2004)
2C1	tPSA, thK2	fPSA-N, fPSA(2C1)[b], tPSA(2C1)[b] tracer in all	IV	(Pettersson *et al*., 1995)
2E9	tPSA	thK2 blocker	I-III	(Lilja *et al*., 1991)
4D4	fPSA-I PSA-ACT	fPSA-I tracer, fPSA-N blocker	I-IV	(Nurmikko *et al.*, 2000)
5A10	fPSA	fPSA tracer	I-IV	(Lilja *et al*., 1991)
5A10 Fab	fPSA	fPSA-I, fPSA-N, fPSA(2C1)[b] capture in all	I-IV	(Eriksson *et al*., 2000)
5F7	tPSA	thK2 blocker	I-III	(Nurmikko *et al.*, 2000)
5H6	tPSA	thK2 blocker	I-III	(Nurmikko *et al.*, 2000)
6H10	thK2 (10% tPSA)[a]	thK2 capture, fhK2 tracer	I-III	(Becker *et al.*, 2000a)
7G1	tPSA, thK2	thK2 tracer	I-III	(Nurmikko *et al.*, 2000)
H117	tPSA, thK2	tPSA, tPSA(2C1)[b] fPSA capture in all	I-IV	Abbot, USA Characterized in (Eerola *et al.*, 1997; Piironen *et al.*, 1998)
H50	tPSA, thK2	tPSA tracer	I-III	Abbot, USA Characterized in (Eerola *et al.*, 1997; Piironen *et al.*, 1998)

[a] Mab 6H10 has 5% cross-reaction with tPSA (with 20 times lower affinity)
[b] tPSA(2C1) and fPSA(2C1) are assays made in parallel with fPSA-N assay

4.2.2 Antibody fragments

Preparation of F(ab´)$_2$ fragments

To reduce the immunoassay interference the Mabs 11B6 and 6H10 were digested to F(ab')$_2$ fragments with bromelain treatment (**I**) which results in removal of the Fc part of the Mab. The bromelain containing ID-Diluent 1 (Diamed, USA) was added to the Mab in 0.5 M Tris-HCl, pH 7.0, 1 M NaCl, 30 mM EDTA (32 µL of Diluent for each mg of Mab) and incubated for 2 h at +37 °C. The reaction was stopped by adding 1/10 reaction volume

of freshly made 0.2 M ethylene maleimide. The $F(ab')_2$ fragments were purified as described in chapter 4.2.3.

Recombinant 5A10 Fab fragment

The recombinant 5A10 Fab fragment was cloned from the monoclonal hybridoma cell line as described by Eriksson *et al*. (Eriksson *et al*., 2000). The Fab fragment containing expression vector pKK incorporated C-terminal cysteine and hexahistidine tag to the Fab fragment which provided the purification site and enabled the site-specific biotinylation.

The recombinant 5A10 Fab fragment was expressed into the periplasmic space of *Escherichia coli* strain RV308 in a 4 L batch fermentation (BioFlow3000 fermentor, New Brunswick Scientific, USA) in SB medium (30 g/L tryptone, 20 g/L yeast extract, 10 g/L MOPS, pH 7.0) supplemented with 0.1 g/L ampicillin, 0.2% glucose and 2 mM $MgSO_4$. The inoculation was done when the cells were at exponential growth phase. The cells were grown overnight at +26 °C to OD600 of 4 after which the production of the Fab was induced by adding isopropyl β-D-1-thiogalactopyranoside (IPTG) to 100 μM. The growth was monitored by OD600 and oxygen consumption and the cells were harvested by centrifugation when the growth started to decrease. The cell pellets were stored at –70 °C prior to purification.

The purification was done essentially as previously described (Korpimaki *et al*., 2004). Briefly, the cells were lysed by Tris buffer extraction based on osmotic shock. The Fab fragment was purified first with cation exchange chromatography with Streamline SP 25 (GE Healthcare, USA) and then using immobilized metal affinity chromatography (Ni-NTA Agarose, Qiagen, Germany). The biotinylation of the free cysteines at the Fab C-terminus was done while the Fab fragments were bound in the Ni-NTA column by circulating 400 μM EZ-Link PEO-maleimide activated biotin (Thermo Scientific, USA) through the column for 2 h. Finally, the buffer was changed to TSA buffer (50 mM Tris-HCl, pH 7.75, 150 mM NaCl, 0.5 g/L NaN_3) with desalting column (GE Healthcare) and bovine serum albumin (BSA) was added to 1 g/L to enhance stability.

4.2.3 Purification, biotinylation and labeling of Mabs and antibody fragments

The Mabs produced by the hybridoma cells and the enzymatically digested $F(ab')_2$ fragments were purified with Protein G column (GE Healthcare Life Sciences, UK) which was equilibrated with 20 mM sodium phosphate buffer, pH 7.5. Elution of the bound antibody or $F(ab')_2$ fragment was done with 0.1 M glycine, pH 2.7. The pH was immediately neutralized by collecting the fractions on 1/10 of the fraction volume of 1 M Tris-HCl, pH 9. The antibody or $F(ab')_2$ fragment containing fractions were pooled by their absorbance at 280 nm and the buffer was changed to 0.9% NaCl before further modifications. The purity of the $F(ab')_2$ fragments were verified by running a sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).

The antibodies and their $F(ab')_2$ fragments were biotinylated using 40–60-fold molar excess of biotin isothiocyanate in 50 mM $NaHCO_3$, pH 9.8. The reaction was incubated at room temperature for 4 h after which the excess biotin was removed by changing the

buffer twice with desalting columns (GE Healthcare) equilibrated and eluted with TSA buffer, pH 7.75. To enhance the stability BSA was added to 1 g/L.

The antibodies and their $F(ab')_2$ fragments were labeled with 50–200-fold molar excess of N1 europium(III) chelate (N1-(4-isothiocyanatobenzyl)diethylenetriamine-N1,N2,N3,N4-tetracis(acetic acid) in 50 mM $NaHCO_3$, pH 9.8. The reaction was incubated overnight at +4 °C (room temperature for Mab 7G1) after which the unreacted chelate was removed by gel filtration with Superdex 200 HP column (GE Healthcare) equilibrated and run with TSA, pH 7.75. The fractions containing the antibody were pooled by the absorbance of the fractions at 280 nm and BSA was added to 1 g/L.

4.2.4 PSA and hK2

The recombinant proPSA and ek-hK2 (mutated pro-sequence to enhance stability) used as immunoassay standard material were produced using a baculovirus expression system *Trichoplusia ni* (High Five) as described previously (Lovgren et al., 1997; Rajakoski *et al*., 1997). The proteins were purified with affinity chromatography. AffiGel 10 support matrix (Bio-Rad, USA) was coupled with the monoclonal antibody 5A10 for proPSA and 11B6 for ek-hK2 purification. The columns were equilibrated and run with 50 mM Tris, pH 7.2, 0.5 M NaCl and the elution was done with 0.2 M glycine, pH 2.5. The fractions were neutralized by collecting the eluted proteins on 1/10 of the fraction volume of 1 M Tris-HCl, pH 9.

The dilutions of the standard material were made in TSA, pH 7.75, with 1 g/L protease-free BSA. The concentrations were calibrated against World Health Organization (WHO) free PSA standard (Rafferty *et al*., 2000) using the tPSA immunoassay (chapter 4.3.3) which recognizes PSA and hK2 with equal affinity (Lovgren *et al*., 1995). The concentration range was from 0.006 ng/mL to 570 ng/mL (6 dilutions) for proPSA and from 0.0034 ng/mL to 3.4 ng/mL (7 dilutions) for ek-hK2. For the tPSA and fPSA assays performed in Malmö University Hospital the standard material included in the Delfia Prostatus PSA F/T Dual Assay kit (Perkin-Elmer Life Sciences, USA) was used.

The seminal plasma PSA pools A, B, C, D and E, used for fPSA-N immunoassay evaluation, were a kind gift from Professor Ulf-Håkan Stenman (Department of Clinical Chemistry, University of Helsinki, Helsinki, Finland). They were fractionated by anion exchange chromatography as described by Zhang *et al*. (Zhang *et al*., 1995). The pools A and B contained only intact PSA with different glycosylation (Mattsson *et al.*, 2008), C and D mostly internally cleaved forms (20% and 10% of intact PSA respectively) and pool E was 95% internally cleaved PSA (5% of intact PSA).

4.3 Immunoassays

4.3.1 General immunoassay protocol

All immunoassays were conducted on streptavidin coated 96-well microtitration plates (Innotrac Diagnostics, Finland or Kaivogen Oy, Finland) either with 1235 Automatic

immunoassay system AutoDelfia (Perkin-Elmer Life Sciences) or manually. The following basic protocol was used unless otherwise stated. The assays were performed in three steps with 1 h incubation at room temperature with slow shaking. The plates were washed twice after capture antibody and sample incubations and four times after label incubation. The enhancement solution (200 µL/well) was incubated 5 to 10 min at room temperature with slow shaking before time-resolved europium fluorescence measurement by AutoDelfia or with Victor 420 Multilabel Counter (Perkin-Elmer Life Sciences). The concentrations of the unknown samples were calculated using MultiCalc software version 2.52 (Perkin-Elmer Life Sciences). The assay buffers used in each assay are described in the following chapter (4.3.2).

4.3.2 Assay buffer

All assays had a common assay buffer containing 50 mM Tris-HCl, pH 7.75, 150 mM NaCl, 0.05% NaN_3, 0.01% Tween 40, 0.05% bovine gammaglobulin, 20 µM diethylene triamine pentaacetate, 0.5% bovine serum albumin and 20 mg/L cherry red. This buffer was used without supplements for the capture antibody dilutions. The bovine IgG reduces some of the unspecific reactions but for sample and label incubations the buffer was supplemented with different blocking agents to further reduce the heterophilic interference originating from the sample matrix.

For total and free PSA immunoassays the assay buffer was supplemented with 5 µg/mL native mouse IgG, 5µg/mL MAK-33 monoclonal antibody (Roche Applied Science, Switzerland), 5 µg/mL heterophilic antibody blocking agent HBR-2 (Scantibodies Laboratory, USA) and 100 U/mL heparin.

For the original thK2 assay the assay buffer was supplemented with 10 mg/L native mouse IgG, 5 mg/L denatured mouse IgG and 10 mL/L mouse serum and for the fhK2 assay with 10 mg/L native mouse IgG and 25 mg/L denatured mouse IgG. For the original fPSA-I assay the assay buffer was supplemented with 10 mg/L native mouse IgG and 5 mg/L denatured mouse IgG. The composition was changed during the assay optimization process as described below.

In the study of assay interference (**I**) the initial screening of samples was performed with assay buffer without supplements but the above mentioned blockers as well as mouse serum and unspecific monoclonal antibodies were tested both in their native and heat-denatured forms and in different combinations during the optimization process. The heat-denaturation of the different blockers was performed at 63 °C for 30 min. The optimized thK2, fhK2 and fPSA-I assays were made with an assay buffer supplemented with 25 mg/L denatured mouse IgG. This buffer was also applied to fPSA-N assays as well as the tPSA(2C1) and fPSA(2C1) assays performed in parallel with the fPSA-N assay.

4.3.3 Total and free PSA immunoassays (I–IV)

The tPSA and fPSA concentrations were measured using in-house immunoassays (**I, II, IV**) except for the measurements made at Lund University Hospital in Malmö which were performed using Delfia Prostatus PSA F/T Dual Assay (Perkin-Elmer Life Sciences) (**III**).

The capture antibody for both assays was biotinylated H117 (300 ng/well in 100 µL of assay buffer in each well). The proPSA standards and samples were added in 25 µL volume on 100 µL of assay buffer in each well. The europium labeled tracers were H50 Mab for tPSA assay and 5A10 Mab for fPSA assay (both 100 ng/well in 200 µL of assay buffer per well).

4.3.4 Intact fPSA immunoassay (I–IV)

Recombinant 5A10 Fab fragment, which was site-specifically biotinylated to its C-terminal cysteine, was used as the capture antibody (150 ng/well in 100 µL of assay buffer). The proPSA standards and samples were added in 50 µL volume on 100 µL of assay buffer per well. The europium labeled Mab 4D4 was used as tracer (200 ng/well in 200 µL of assay buffer per well).

The original assay protocol used for measuring the Göteborg cohort (**III**) was performed with a different buffer (see 4.3.2). The capture antibody was the intact, biotinylated 5A10-Mab (200 ng/well in 200 µL assay buffer), and the sample incubation was done with 0.5 µL of mouse serum in 100 µL of assay buffer in each well for 2 h. The label incubation and measurement were done as in the optimized assay.

4.3.5 Nicked fPSA immunoassay (IV)

Calculated fPSA-N concentrations

The calculated concentrations of fPSA-N were used in publications **II-IV**. The calculation was done by subtracting the fPSA-I concentration from fPSA concentration. If the fPSA-I concentration was higher than fPSA concentration the fPSA-N concentration was set to 0.001 ng/mL and the I/F PSA ratio to 100% for statistical analysis.

*Interference in fPSA-N immunoassay (**unpublished**)*

Based on the interference problems seen in fPSA-I immunoassay it was hypothesized that interference would also be a similar problem in fPSA-N immunoassay as the capture antibody is the same. This was verified by measuring three pools of female samples (consisting of 9, 10 and 12 samples, **Table 5**.), known to produce falsely high signals in fPSA-I immunoassay, with fPSA-N immunoassay and fPSA(2C1) immunoassay made in parallel with a similar protocol with both Mab and Fab capture antibodies with assay buffer supplemented with heat-denatured mouse IgG.

fPSA-N immunoassay and fPSA(2C1) and tPSA(2C1) immunoassays made in parallel

The fPSA-N immunoassay principle is illustrated in **Figure 8A**. The biotinylated recombinant 5A10 Fab fragment was used as the capture antibody (150 ng in 25 µL of assay buffer per well) and the proPSA standards and samples were added in 50 µL volume on top of 50 µL of assay buffer and incubated for 2 h at room temperature with slow shaking. After two washes, 5000 ng of 4D4 Mab was added in 50 µL of assay buffer in each well to block all intact PSA. The plate was sealed with a tape and incubated overnight at +4 ºC without shaking. After incubation without a washing step the europium

labeled 2C1 tracer Mab was added (100 ng in 50 µL of assay buffer per well) and the incubation was continued for 4 h at +4 ºC. After four washes the measurement was made as described above (chapter 4.3.1).

In order to obtain fPSA and tPSA concentrations, which would be comparable to fPSA-N concentrations, similar protocols were used to measure the concentrations of fPSA and tPSA in parallel with fPSA-N immunoassay (see **Figure 8B** and **C**). The biotinylated capture antibodies were the recombinant 5A10 Fab fragment (150 ng/well) in fPSA(2C1) assay and H117 in tPSA(2C1) assay (300 ng/well), both applied in 25 µL of assay buffer in each well. The sample incubation was done as in fPSA-N assay but instead of blocker Mab 4D4, only assay buffer was used. The tracer in both assays was europium labeled 2C1 Mab as in the fPSA-N assay.

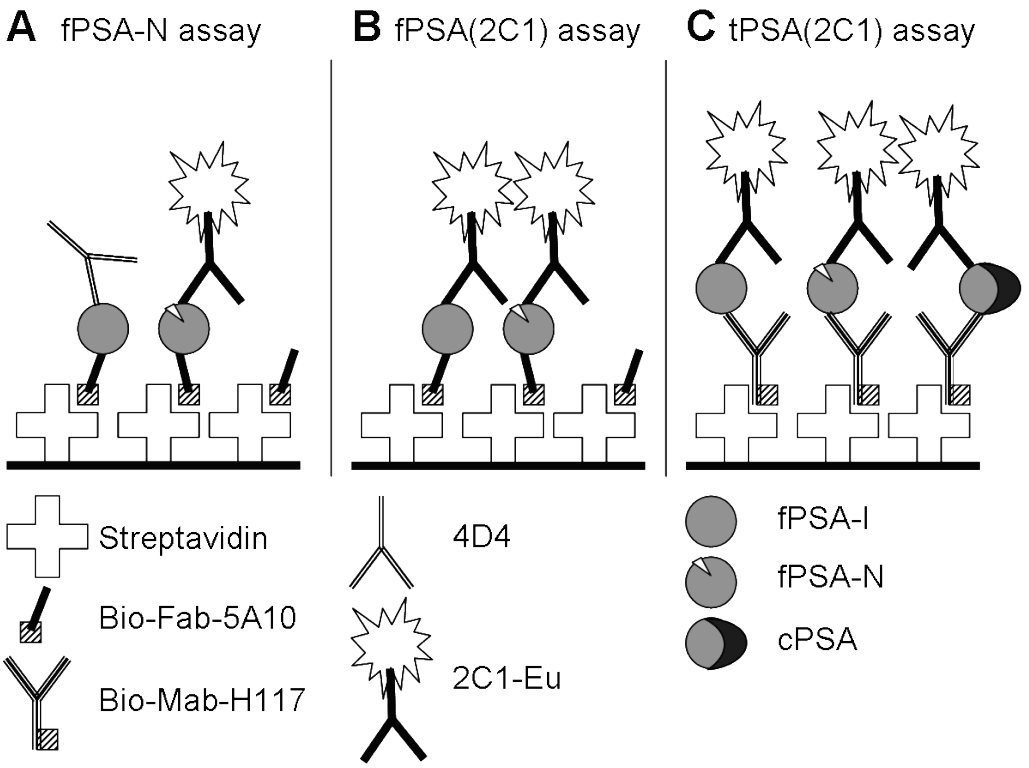

Figure 8. The principle of the direct fPSA-N immunoassay and the assays for fPSA and tPSA measurements. **A**) fPSA-N assay. **B**) fPSA(2C1) the standard curve of which was also used for calculating fPSA-N concentrations (see text for details). **C**) tPSA assay. cPSA = complexed PSA. The fPSA(2C1) and tPSA(2C1) assays were made in parallel with the same overall protocol to obtain comparable concentrations (**IV, unpublished**).

The fPSA(2C1) immunoassay standard curve was used to calculate the fPSA-N concentrations because the standard material, intact proPSA, was blocked in the fPSA-N assay. The standard curve in the fPSA-N assay was used to calculate the efficiency of the

4D4 blocking. The specific signal of each calibrator dilution in fPSA-N assay was divided by the corresponding signal in the unblocked fPSA(2C1) assay, the resulting ratio was then subtracted from 1 and multiplied by 100% to obtain the blocking efficiency for the particular calibrator dilution in each run. Because the blocking was not perfect, the average blocking efficiency of all calibrator dilutions was used to correct the fPSA-N concentrations for this cross-reaction with fPSA-I.

4.3.6 Total and free hK2 immunoassays (I–III)

Biotinylated 6H10-F(ab')$_2$ fragment was used as the capture antibody (300 ng in 100 µL of assay buffer per well). The recombinant ek-hK2 standards and samples were added in 100 µL volume on 100 µL of assay buffer per well with PSA specific antibodies 2E9 (1000 ng/well), 5F7 (1000 ng/well) and 5H6 (500 ng/well). The blocker antibodies had epitopes overlapping with the epitopes of the capture and tracer antibodies minimizing the cross-reaction of PSA. The europium labeled tracer 7G1 Mab was added (100 ng) with blocker Mab 5H6 (500 ng) in 200 µL of assay buffer in each well. The measurement was made as described above.

The original thK2 assay was made as the optimized assay described above except that the buffer described in chapter 4.3.2 was used, capture antibody was the intact, biotinylated 6H10-Mab (300 ng/well in 100 µL assay buffer) and the sample incubation was 2 h instead of 1 h.

Biotinylated 11B6-F(ab')$_2$ fragment was used as the capture antibody (300 ng in 100 µL of assay buffer per well). The recombinant ek-hK2 standards and samples were added in 100 µL volume on 100 µL of assay buffer per well. The tracer was europium labeled 6H10-F(ab')$_2$ fragment (100 ng in 200 µL of assay buffer per well). The measurement was made as described above.

The original fhK2 assay differed from the optimized protocol by the buffer (see chapter 4.3.2), and by the capture and tracer antibodies being intact Mabs instead of F(ab')$_2$ fragments. Furthermore, for the capture, biotinylated 11B6-Mab was used 300 ng/well in 100 µL of assay buffer and the sample incubation was 2 h instead of 1 h in the optimized assay.

4.4 Statistical analyses

Statistical calculations were performed with R software, version 2.0.1 (R Development Core Team, 2005) (**II**), GraphPad Prism, version 4.03, for Windows (GraphPad Software, San Diego CA) (**II** and **IV**) or Stata 10.0/11.0 (StataCorp, College Station, TX) (**III**). The two-sided P-values < 0.05 were considered to be statistically significant. The correlations of different analytes and clinical or pathological attributes were studied with Spearman's rank correlation (**II, III**). The comparisons between different groups of patients were made from log-transformed marker concentrations with analysis of variance (**II**). Multivariable logistic regression was used to fit the predictive models based on kallikrein and clinical measurements in both training and validation sets (cohort randomly divided in a 1:3 ratio in Rotterdam and 2:1 in Göteborg, respectively) (**III**). tPSA and fPSA were

entered into the logistic models using restricted cubic splines to account for nonlinearity. The comparison of calculated and measured fPSA-N concentrations in patient samples was made with Deming (type II) regression analysis (**IV**).

5 SUMMARY OF RESULTS

5.1 Removal of assay interference

5.1.1 Interference rate (I)

Female (n=1092) and male (n=957) routine heparin plasma samples were screened to select samples with interference. Female samples were measured with fPSA-I and fhK2 assays and male samples with fPSA-I, fPSA, fhK2 and thK2 assays, all in singlicates. The assay buffer contained no other scavenger antibodies in addition to the bovine IgG. The interference was defined as fPSA-I or fhK2 concentrations above 0.01 ng/mL in female samples and ratios of I/F PSA and F/T hK2 above 1 in male samples. The observed interference rates are shown in **Table 7**.

Table 7. Number of heparin plasma samples exhibiting falsely high signals in initial screening. "Both" is the number of samples having interference in both fPSA-I and hK2 assays (**I**).

Female samples n=1092	**n**	**%**	**Male samples n=957**	**n**	**%**
fPSA-I > 0.01 ng/ml	350	32%	I/F PSA > 1	165	17%
fhK2 > 0.01 ng/ml	427	39%	F/T hK2 > 1	335	35%
Both	270	25%	Both	108	11%
Total interference	507	46%	Total interference	392	41%

5.1.2 Blocking components in the assay buffer (I)

Several different blocking agents were tested for their capability to decrease the interference. The heat-denatured mouse IgG as 25 mg/L concentration was the best among the tested blockers and their combinations. In some samples the presence of blockers caused the false signal to rise even higher. This was especially evident when the blockers were in their native form (native mouse IgG, mouse serum or untreated MAK-33). Despite adding the blockers into the assay buffer about 30% of the pre-selected samples still had interference.

5.1.3 Optimization of the capture and tracer antibodies (I)

Several samples were depleted during the optimization process and therefore the number of tested samples decreased in each step. The Mabs were changed to $F(ab')_2$ or Fab fragments to reduce the interference caused by the interfering molecules attaching to the Fc-part of the Mab.

Female samples

Of the 78 samples having interference in fPSA-I assay with Mabs 33 (42%) had signal levels above 0.01 ng/ml after changing the capture Mab to recombinant Fab fragment and only 4 (5%) samples had elevated signals when the buffer with 25 mg/L denatured mouse

IgG was combined with the Fab-based assay. Changing both capture and tracer Mab in the fhK2 assay decreased the number of interference samples to 3 in 157 (2%) previously high signal samples. The use of 25 mg/L of denatured mouse IgG decreased the fhK2 signals but 3 (2%) samples remained above 0.01 ng/mL.

Male samples

Male samples having I/F PSA or F/T hK2 ratios above 1.5 were considered to have high interference and were used in optimization to ensure that the elevation in signal level is caused by the interference and not by assay variation. Of the 957 samples, high interference was found in 183 (19%) samples. The I/F ratio was high in 42 samples, the F/T hK2 ratio was high in 90 samples and both ratios were high in 51 samples.

Changing the fPSA-I assay capture Mab to Fab fragment reduced the number of samples with I/F PSA ratio > 1 to 11/47 (23%) and using the assay buffer with 25 mg/L denatured mouse IgG further reduced it to only 2/47 (4%) samples with interference.

Using F(ab')$_2$ fragments in fhK2 assay reduced the number of samples with F/T hK2 > 1 to 25/140 (18%) and with the buffer containing 25 mg/l denatured mouse IgG the number was 15/96 (16 %). When also the capture Mab of thK2 assay was changed to F(ab')$_2$ fragment the F/T hK2 ratio was above 1 in 9/96 (9 %) samples and combining both optimized assays with the optimized assay buffer containing the mouse IgG further reduced the number of interference samples to only 3/96 (3%).

Based on these results the optimized assay formats were as stated in **Table 8** and in chapter 4.3. These assays were used in further studies.

Table 8. The screening and optimized assay formats (**I**).

	Screening assay			**Optimized assay**		
	fPSA-I	**fhK2**	**thK2**	**fPSA-I**	**fhK2**	**thK2**
Capture and tracer antibodies						
Biotinylated capture	Mab	Mab	Mab	Fab	F(ab')$_2$	F(ab')$_2$
Eu-labeled tracer	Mab	Mab	Mab	Mab	F(ab')$_2$	Mab
Blocking antibodies in the assay buffer						
Bovine IgG	Yes	Yes	Yes	Yes	Yes	Yes
Denatured mouse IgG	No	No	No	Yes	Yes	Yes

5.1.4 Evaluation of optimized assays (I–III)

The optimized assays were evaluated by measuring EDTA plasma, heparin plasma and serum samples with the original and optimized assays (**Table 8**). The results are shown in **Table 9**.

The number of samples having I/F PSA and F/T hK2 ratios above 1 was reduced significantly when the optimized assays were used. Also, the magnitude of the interference was decreased. In the non-optimized fPSA-I assay the concentrations in interference samples were generally up to 30 times higher than the fPSA concentrations, while in the optimized fPSA-I the concentrations in the remaining interference samples were only up to 3 times higher than the fPSA concentrations. The change in the hK2 assays was similar; the F/T hK2 ratios were up to 145 in non-optimized assays while in the optimized assays only 6-fold difference was seen. In addition to this, the remaining interference was in the low concentration range near the limit of quantification in all assays.

The number of interfering samples was low also in the later studies (**II**, **III**) as shown in **Table 9**. Notably, in the ERSPC Rotterdam cohort the I/F PSA was above 1 in only 6 out of 2914 serum samples (0.2%). Only thK2 was measured from the samples so no data is available for the hK2 assay interference rate in this cohort.

Table 9. Evaluation of the optimized assays and interference rate in sample panels measured in the optimized assays. See details of the sample panels from Table 5 (**I–III, unpublished**).

Sample panel	**Sample matrix**	**Total number**	**Mab-based assay**		**Fab- or F(ab')$_2$-based assay**	
		n	**n**	**%**	**n**	**%**
			I/F PSA > 1			
Turku evaluation (**I**)	serum	171	24	**14**	5	**3**
	heparin	122	19	**16**	2	**2**
	EDTA	94	9	**10**	1	**1**
Turku, clinically defined rp patients (**II**)[a]	heparin	111			0	**0**
	EDTA	198			5	**3**
Rotterdam (**III**)	serum	2914			6	**< 1**
			F/T hK2 > 1			
Turku evaluation (**I**)	serum	171	14	**8**	7	**4**
	heparin	138	21	**15**	8	**6**
	EDTA	111	28	**25**	5	**5**
Turku, clinically defined rp patients (**II**)[a]	heparin	111			4	**4**
	EDTA	198			8	**4**

[a] rp, radical prostatectomy

5.1.5 Interference in fPSA-N assay (unpublished)

The effect of interfering factors on fPSA-N immunoassay, described in chapter 5.4 (**IV**), was tested using three pools of female heparin plasma known to have interference in fPSA-I assay based on measurements made at Malmö University Hospital. Two out of

three pools showed a signal above 0.02 ng/mL in fPSA-N assay and all three in fPSA(2C1) assay made in parallel when the Mab capture antibody was used. When the capture Mab was changed to Fab fragment, all three pools were below 0.02 ng/mL in fPSA-N assay and only one pool had a concentration of 0.05 ng/mL in fPSA(2C1) assay. In further fPSA-N, fPSA(2C1) and tPSA(2C1) assays the Fab fragment was used as the capture antibody and 25 mg/L heat-denatured mouse IgG was added to the assay buffer.

5.2 fPSA isoforms in radical prostatectomy patients (II)

The clinical value of preoperative fPSA-I and calculated fPSA-N in predicting the pathological features of prostate cancer was studied in 309 men scheduled to undergo radical prostatectomy at Turku University Hospital. The median fPSA-I concentration was 0.42 ng/mL and it constituted 59.8% (median value) of fPSA and 5.7% of tPSA while fPSA-N median concentration was 0.28 ng/mL constituting 40.2% of the fPSA and 3.6% of the tPSA in the plasma. Median fPSA concentration was 0.70 ng/mL and the median F/T PSA ratio was 9.7%.

The tPSA concentrations between 4 and 10 ng/mL have been considered to represent a so called "gray zone" because the cancer risk estimation is even more difficult than with the extremely high or low concentrations (Catalona *et al.*, 1998; Catalona *et al.*, 1994). Therefore, we also analysed the subgroup of patients with tPSA concentrations below 10 ng/mL.

Generally, the ratios of N/T PSA and F/T PSA were lower in patients with more advanced pathological features. These ratios had the strongest negative correlations with pathologic stage, WHO grade and Gleason score, though the correlations were modest (between –0.205 and –0.262). In addition to N/T and F/T PSA ratios, also fPSA-N as the only single marker correlated with Gleason score in patients with tPSA < 10 ng/mL.

The individual markers could not separate different groups based on pathological TNM stage. I/T PSA could statistically separate pT2b group from pT3a group and N/T PSA pT3a and b groups from each other and F/T PSA was statistically different in both cases (**Figure 9**). I/T PSA was able to separate the two groups also in patients with tPSA < 10 ng/mL. When the groups were combined to organ-confined (pT2) and non-organ-confined (pT3) groups, all three ratios and tPSA as the only single marker had statistically significant p-values (all < 0.01). In patients with tPSA < 10 ng/mL the difference remained significant only for tPSA and F/T PSA.

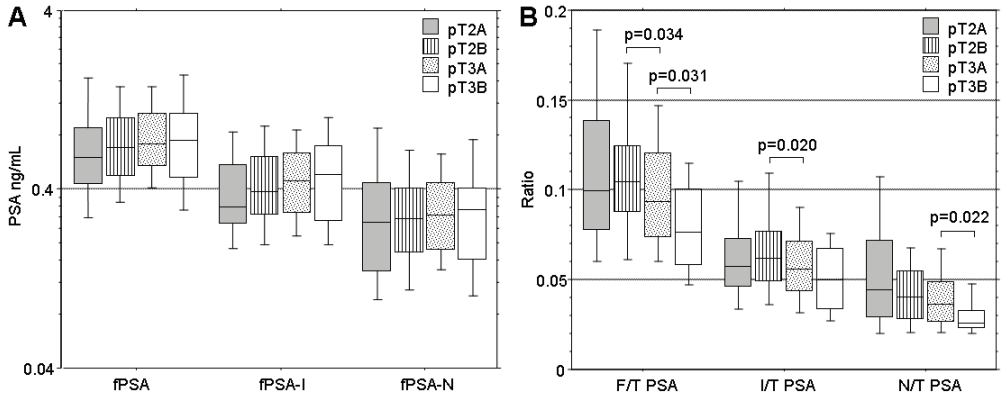

Figure 9. Box plot distributions of PSA isoform concentrations (**A**) and their ratios (**B**) in prostate cancer patients with different pathological TNM stage (**unpublished**). Boxes show median (line inside the box) and 25th and 75th percentiles (limits of the box). Whiskers show 10th and 90th percentiles. **A**) None of the markers could statistically separate different patient groups. Number of patients in the groups: pT2A, n = 52; pT2B, n = 94; pT3A, n = 130 and pT3B, n = 25. **B**) All ratios were able to differentiate at least two consecutive groups (the analysis of variance p-values < 0.05 shown). Number of patients in the groups: pT2A, n = 50; pT2B, n = 89; pT3A, n = 127 and pT3B, n = 25.

The ratios were also able to statistically separate patients with different grades of cancer. N/T PSA was able to statistically separate all WHO groups, while F/T PSA was significantly different only in groups 2 and 3. Both ratios were able to separate patients with Gleason score < 7 from patients with Gleason score 7 as shown in **Figure 10**. N/T PSA, I/T PSA and fPSA-N as the only single marker were able to separate the groups also in patients with tPSA < 10 ng/mL. Furthermore, the trend of the decreasing median ratios with increasing Gleason score is seen in **Figure 10**.

It has been suggested that the prognosis of Gleason score 7 patients whose primary Gleason grade is 3 or lower is better than of the patients with primary Gleason grade 4 or higher (Lilleby *et al*., 2001; Steinberg *et al*., 1997). We divided the patients with Gleason score 7 into two new groups: The group 7a included the patients with primary Gleason grade ≤ 3 and the group 7b included the patients with primary Gleason grade ≥ 4. These two groups were further combined with other patients to create a low-grade group with Gleason scores from 3 to 7a and a high-grade group with Gleason scores from 7b to 9. tPSA and all ratios, except for I/F PSA, were able to differentiate the groups. In patients with tPSA < 10 ng/mL only fPSA-N concentration and F/T PSA were different in each group. N/T PSA had a borderline significant p-value of 0.052.

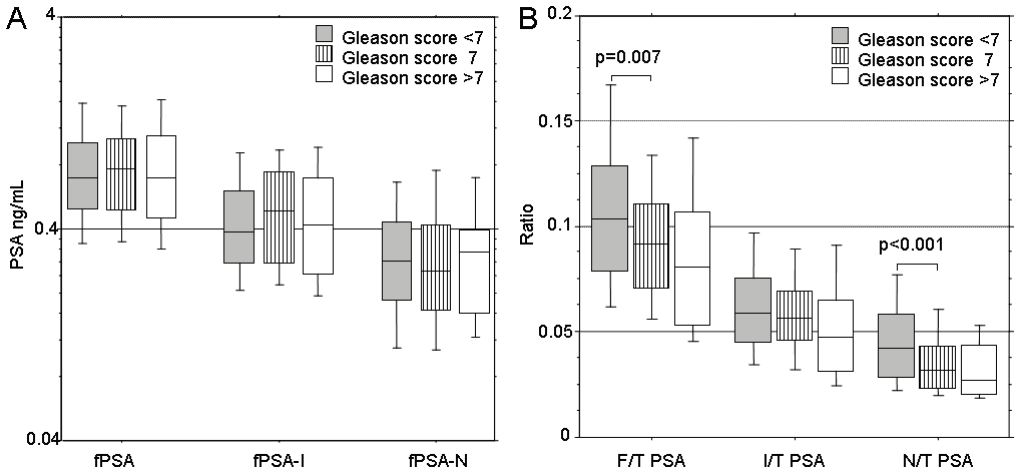

Figure 10. Box plot distributions of fPSA isoforms (**A**) and their ratios (**B**) in prostate cancer patients with different Gleason scores (**unpublished**). Boxes show median (line inside the box) and 25th and 75th percentiles (limits of the box). Whiskers show 10th and 90th percentiles. **A**) None of the markers could statistically separate different patient groups. Number of patients in different groups: Gleason score < 7, n = 190; Gleason score 7, n = 82 and Gleason score > 7, n = 36. **B**) F/T PSA and N/T PSA could separate patients with Gleason score < 7 from patients with score 7 (the analysis of variance p-values < 0.05 shown). Number of patients in different groups: Gleason score < 7, n = 182; Gleason score 7, n = 78 and Gleason score > 7, n = 35.

5.3 Kallikrein panel and prostate volume (III)

A previously developed statistical model based on the measurement of tPSA, fPSA, fPSA-I and thK2 concentrations was shown to be a strong predictor of biopsy outcome in men with elevated tPSA (Vickers *et al.*, 2010a; Vickers *et al.*, 2008). In the current study, the effect of adding digital rectal examination (DRE) and prostate volume measured by transrectal ultrasound into the model was studied. The laboratory based model included age and the kallikrein panel while the clinical model included age, tPSA, DRE and prostate volume. The models had AUCs of 0.766 and 0.763, respectively, for prostate biopsy outcome in Rotterdam and 0.809 and 0.774 in Göteborg (**Table 10**) The AUCs for predicting high-grade cancer were slightly higher than for any cancer in the Rotterdam cohort and slightly lower in the Göteborg cohort. Adding clinical measures to the kallikrein panel increased the predictive accuracy but the increase was statistically significant only in the Rotterdam cohort (**Table 11**).

Furthermore, the correlations of the full model and the individual markers with prostate volume were studied. The full model showed a correlation of 0.60 and 0.57 (Spearman's rank correlation coefficient) in the Rotterdam and Göteborg cohorts, respectively. Of the individual markers, fPSA-N had the highest correlation of 0.55 and 0.41 in the Rotterdam and Göteborg cohorts, respectively (**Table 12**).

Table 10. Predictive accuracy for prostate cancer of models built on the training set and applied to the validation set (**III**).

Model	**Rotterdam**		**Göteborg**	
	Any cancer AUC	High-grade cancer AUC	Any cancer AUC	High-grade cancer AUC
A Kallikrein panel (age, tPSA, fPSA, fPSA-I, thK2)	0.766	0.847	0.809	0.786
B Clinical model (age, tPSA, DRE, TRUS volume)	0.763	0.846	0.774	0.681
C Full laboratory model: Kallikrein panel (**A**) + DRE	0.778	0.856	0.810	0.802
D Laboratory plus clinical model: Kallikrein panel (**A**) + DRE + TRUS volume	0.792	0.860	0.826	0.802

Table 11. Comparison of the differences in predictive accuracy between the models (see **Table 10**). Data are given as difference in AUCs and p-value for the difference. (**III**)

Comparison	**Rotterdam**		**Göteborg**	
	Any cancer Difference (p-value)	High-grade cancer Difference (p-value)	Any cancer Difference (p-value)	High-grade cancer Difference (p-value)
A vs. B	-0.003 (p=0.8)	-0.001 (p=1)	-0.035 (p=0.3)	-0.105 (p=0.2)
C vs. A	0.012 (p=0.033)	0.009 (p=0.17)	0.001 (p=0.9)	0.016 (p=0.3)
C vs. B	0.015 (p=0.15)	0.010 (p=0.075)	0.036 (p=0.3)	0.121 (p=0.3)
D vs. C	0.014 (p=0.002)	0.004 (p=0.2)	0.016 (p=0.14)	0.000 (p=0.9)

Table 12. Correlation between TRUS volume and kallikrein isoforms. The analyses were performed on the total cohorts. All correlations were statistically significant ($p < 0.05$). (**III**)

Measure	**Rotterdam Spearman's correlation**	**Göteborg Spearman's correlation**
Kallikrein panel	0.60	0.57
fPSA-N	0.55	0.41
fPSA	0.53	0.51
N/T PSA	0.46	0.39
fPSA-I	0.44	0.36
F/T PSA	0.43	0.48
thK2	0.33	0.19
tPSA	0.21	0.15

5.4 Development of the fPSA-N immunoassay (IV)

5.4.1 Analytical performance

The fPSA-N and fPSA(2C1) immunoassays had a common standard curve because intact proPSA was used as the calibrator. This and the standard curve for tPSA(2C1) assay are shown with precision profiles (mean of five different runs) in **Figure 11**. The blocking efficiency of Mab 4D4 in the fPSA-N assay was calculated as average of six calibrator dilutions in five different runs and it was a stable 88% at the linear range of the assay (0.059–55 ng/mL). To correct the fPSA-N concentrations for the 12% cross-reaction with fPSA-I the measured fPSA-N concentrations were multiplied with the correction factor 0.88.

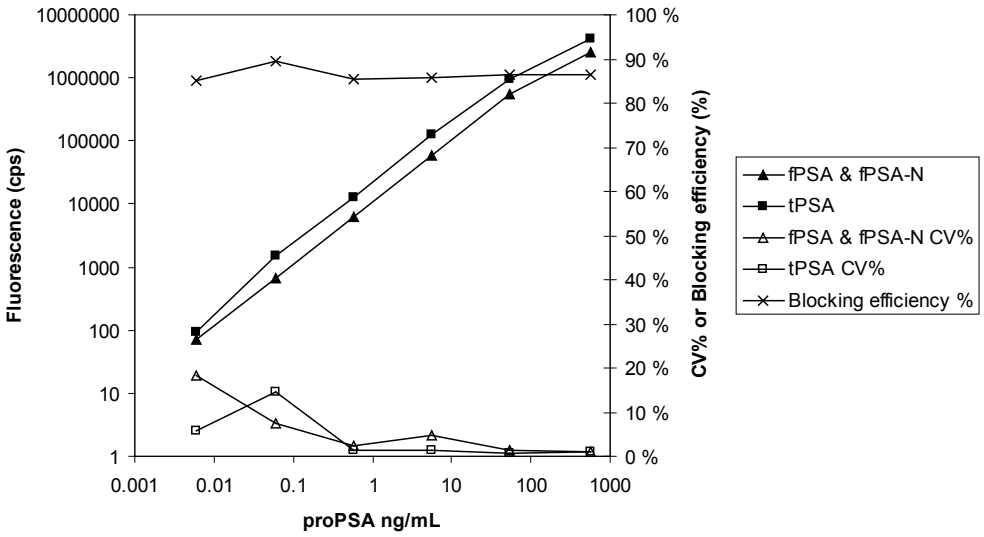

Figure 11. Standard curves (closed symbols) and imprecision profiles (open symbols) for fPSA(2C1) and fPSA-N assays (rectangle) and tPSA(2C1) assay (square) (**IV**). The same standard curve was used for both fPSA(2C1) and fPSA-N assays due to standardization with intact proPSA. Blocking efficiency of fPSA-N assay at different proPSA concentrations is shown with X.

The assay performance characteristics for the developed fPSA-N, fPSA(2C1) and tPSA(2C1) and optimized fPSA-I assay are shown in **Table 13**. The calculation of the analytical and functional detection limits for the fPSA-I assay and for the previously reported fPSA and tPSA assays are based on measurements reported in publication II. The detection limits of fPSA-N and fPSA(2C1) assays were close to the detection limit of the optimized fPSA-I assay. The detection limits of tPSA(2C1) assay were close to the detection limits of the previously published tPSA assay.

Table 13. Assay performance characteristics for the developed fPSA-N, fPSA(2C1) and tPSA(2C1) assay and optimized fPSA-I assay as well as, for comparison purposes, for previously reported fPSA and tPSA assays.

Assay	Publication	Analytical det. lim. ng/mL[a]	Functional det. lim. ng/mL[b]	Recovery from plasma % (range)
fPSA-N	IV	0.016	0.100	95.0 (89.6–145.3[c])
fPSA(2C1)	IV	0.016	0.100	90.7 (84.7–101.4)
tPSA(2C1)	IV	0.006	0.05	94.8 (87.9–108.1)
fPSA-I	II , IV	0.01[d]	0.13	84 (75–96)[e]
fPSA	II , unpublished	0.006[f]	0.03[e]	N.D.[g]
tPSA	II , unpublished	0.006[f]	0.03[f]	N.D.

[a] det. lim., detection limit, calculated as mean of calibration diluent + 2SD
[b] det. lim., detection limit, estimation of lowest concentration with CV% < 20%
[c] A single sample of the five samples measured
[d] Originally reported analytical det. lim. 0.035 ng/mL (Nurmikko *et al.*, 2001)
[e] in serum, from (Nurmikko *et al.*, 2001)
[f] Originally reported analytical det. lim. 0.01 ng/mL and functional det. lim. < 0.1 ng/mL (Lerner *et al*., 1996)
[g] N.D., no data

5.4.2 Detection of different seminal plasma PSA isoforms

Five seminal plasma PSA pools containing differing proportion of intact and nicked PSA (Zhang *et al*., 1995) were measured with fPSA-N, fPSA(2C1) and tPSA(2C1) assays to ensure that the fPSA-N assay measures nicked PSA as specifically as expected. The ratios of fPSA-N and fPSA to tPSA are shown in **Figure 12**. Pools A and B were reported to contain only intact PSA, pools C 20% and D 10% of intact PSA, and pool E only approximately 5% of intact PSA.

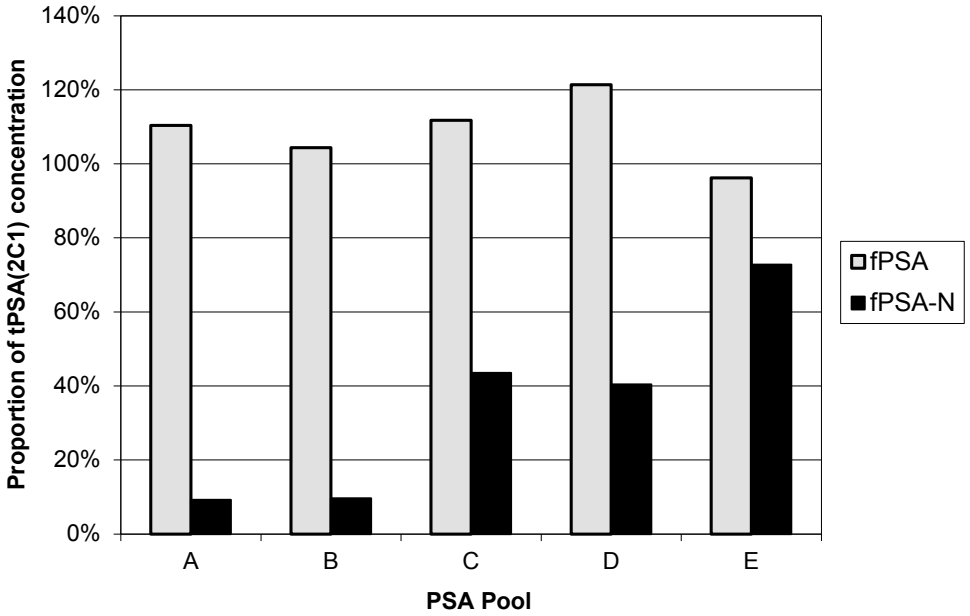

Figure 12. Proportion of fPSA (circle) and fPSA-N (square) to tPSA in seminal plasma PSA pools (**IV**).

5.4.3 fPSA forms in clinical material

The different PSA forms (fPSA-N, fPSA-I, fPSA and tPSA) were at or below the analytical detection limit in all 12 samples from healthy female volunteers. The concentrations and interquartile ranges of the markers for 9 healthy male volunteers, 27 patients with benign prostate condition and 49 prostate cancer patients are shown in **Table 14**.

Table 14. Median and interquartile range (IQR) of different PSA forms and their ratios in healthy males and patients with benign disease and prostate cancer (**IV, unpublished**). The markers were measured with the new assays developed in study **IV**, except for fPSA-I which was measured using the optimized assay (**I**).

Marker median (IQR)	**Healthy n = 9**	**Benign disease n = 27**	**Cancer n = 49**
tPSA (ng/mL)	0.649 (0.432–0.821)	10.34 (4.375–19.56)	5.464 (4.728–10.743)
fPSA (ng/mL)	0.247 (0.151–0.283)	1.385 (0.651–2.771)	0.855 (0.628–1.761)
fPSA-N (ng/mL)	0.050 (0.033–0.064)	0.420 (0.153–0.786)	0.239 (0.131–0.452)
fPSA-I (ng/mL)	0.176 (0.09–0.205)	0.478 (0.284–0.606)	0.550 (0.351–0.914)
F/T PSA (%)	38.5 (35.2–40.8)	19.7 (14.2–27.3)	14.7 (12.4–22.9)
N/T PSA (%)	7.8 (6.8–9.2)	5.1 (4.0–7.2)	3.4 (2.3–4.7)
I/T PSA (%)	27.1 (24.9–27.9)	5.5 (2.8–9.9)	9.4 (6.3–13.0)
N/F PSA (%)	19.9 (18.1–22.4)	27.6 (23.4–36.3)	24.2 (17.4–31.9)
I/F PSA (%)	70.8 (68.2–71.3)	28.7 (21.6–53.6)	64.6 (52.9–74.8)

5.4.4 Comparison of the new assays to previously used methods

In all previous studies the concentration of fPSA-N was calculated by subtracting fPSA-I concentration from fPSA concentration. The calculated and directly measured fPSA-N concentrations of the 76 patients are plotted in **Figure 13A**. Furthermore, the fPSA and tPSA concentrations measured with previously published and new methods in these patients are plotted in **Figure 13B** and **C**. The Deming (model II) regression lines and corresponding equations are also shown in the figures.

The measured fPSA-N concentrations were on average 91% of the calculated values (SD 64%, median 77%, interquartile range 62–96%). fPSA(2C1) concentrations were on average 128% of the concentrations measured with previously used fPSA assay (SD 51%, median 112%, interquartile range 95–145%) while the tPSA(2C1) assay gave a result which was on average 75% of the result from the previously used tPSA assay (SD 14%, median 73%, interquartile range 67–82%).

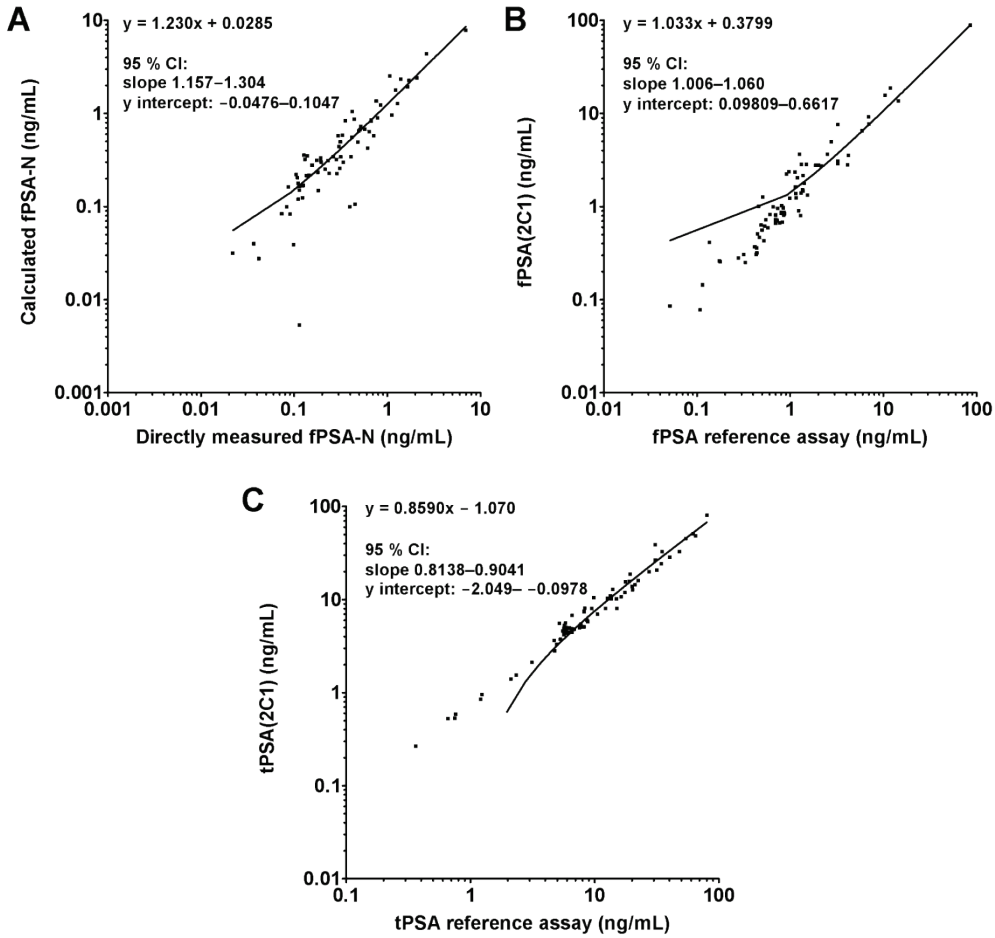

Figure 13. Comparison of the new assays and previously used methods (**IV, unpublished**). Concentrations were measured from 79 samples of men with benign or cancerous prostate condition. Concentrations from different methods are compared by Deming (model II) regression (line and equations). **A**) fPSA-N concentrations determined by directly measuring with the new fPSA-N assay or by calculation (fPSA minus fPSA-N). **B**) fPSA concentrations measured with the new fPSA(2C1) or with the previously used assay (see chapter 4.3.3). C) tPSA concentrations measured with the new tPSA(2C1) assay or with the previously used assay (see chapter 4.3.3).

6 DISCUSSION

6.1 Immunoassays and their performance

Assay interference is a recognized problem for immunoassays and has caused some unfortunate cases of misdiagnosis and unnecessary treatments (Cole *et al.*, 1999; Fritz *et al.*, 2009; Ismail *et al.*, 2002). The interference caused by different antibody-binding molecules in the sample component is often decreased by adding scavenger immunoglobulins to which the proteins should bind instead of the assay antibodies. Even though the results have been good and the scavenger antibodies are used as blocking agents in most immunoassays they do not remove the interference completely.

The prevalence of the assay interference is strongly dependent on the assay construct and the population in question. Despite the use of scavenger antibodies in the assay buffers, our original fhK2 and fPSA-I assays suffered from falsely elevated signals in some samples causing the ratios of F/T hK2 and I/F PSA to be above 1, which is impossible by definition (Nurmikko *et al.*, 2001; Vaisanen *et al.*, 2004; Vaisanen *et al.*, 2006). When 1092 random female and 957 male heparin plasma samples were measured with fPSA-I or fhK2 assays with previously published protocols bovine IgG as the only blocking agent there were falsely elevated signals in 10–40% of the samples (**I**). These rates are within the published range of observed interference rates (see Chapter 2.4.1 for more information) even though they are at the upper end of the range. It is notable that this initial screening was done in singlicates which increases the possibility that assay variation explains part of the falsely elevated signals.

Many of the antibody-binding interfering factors are more likely to bind the Fc part of the antibody molecule and thus, removing the Fc part has been previously used to successfully reduce interference problems (Bjerner *et al.*, 2002; Vaidya and Beatty, 1992). In our assays, changing the capture or tracer Mab to Fab- or $F(ab')_2$-fragment and adding denatured mouse IgG to the assay buffer practically removed or significantly reduced the interference (0–6% of samples exhibiting falsely high signals).The magnitude of the false elevation of the signals was decreased as well. The effects were also seen in large patient sample cohorts measured with the optimized assays.

The original assays have been used to measure many sample panels (Niemelä, 2002; Nurmikko *et al.*, 2001; Steuber *et al.*, 2005; Vaisanen *et al.*, 2004; Vaisanen *et al.*, 2006; Vickers *et al.*, 2008) and the interference may have affected the results. If the prevalence of interfering samples is assumed to be approximately the same in different patient groups (healthy subjects as well as patients with benign conditions and cancer) the presence of interference samples might weaken the association between the markers and prostate cancer and thus cause underestimation of the markers' clinical value. The effect would probably be minor when large panels are measured. However, there is a possibility that the prevalence of the assay interference does differ between the different patient groups. This is not very likely, though, as the results based on the studies using the kallikrein panel show: the overall discrimination was similar in cohorts measured with original and optimized assays (Vickers *et al.*, 2010a; Vickers *et al.*, 2008; Vickers *et al.*, 2010b;

Vickers *et al.*, 2010c). A smaller-scale study (**unpublished**) in which the original and optimized assays were used to measure four sample groups with different tPSA range and F/T PSA ratios (n = 100 in each) shows similar results. The effect of the change in the assay format was minor in each group (results not shown).

A novel observation was that in some samples the blocking antibodies caused a signal elevation instead of a decrease. Even though this was clearly more common when the blocking antibodies were in native form we cannot exclude the possibility that the denatured mouse IgG could also cause false signal elevation in some samples. Alternatively, it is possible that there might have been negative interference in some samples, in which the signals were elevated in the optimized assay but remained at a "logical level". This is difficult to confirm but, if true, could be seen as a desired correction to the result.

The optimized fPSA-I assay had better analytical and functional detection limits than the original assay because the recombinant Fab fragment used as the capture antibody is smaller allowing more dense binding to the streptavidin surface and thus higher antigen binding capacity. Furthermore, the site specific biotinylation enables these fragments to bind to the streptavidin surface in a functional orientation further increasing the capacity. This caused an increase in the signal to background ratio and lead to improved detection limits in the fPSA-I assay. The improved assay performance was also seen in the kallikrein panel when it was applied to recently screened men in both Göteborg and Rotterdam cohorts (Vickers *et al.*, 2010c). The samples from Göteborg were measured with the original assays and the Rotterdam samples with the optimized assays. The predictive accuracy associated with fPSA-I was increased when the optimized assay was used. However, the optimized fhK2 and thK2 assays, based on enzymatically cleaved $F(ab')_2$-fragments, had similar detection limits as the previously used assay protocols (results not shown) and thus the improvement in assay performance in terms of fewer samples with interference may not be as direct and visible as with fPSA-I assay.

The assay interference observed in the fPSA-I assay suggested that the fPSA-N and fPSA(2C1) assays (**IV**) might be prone to similar interference problems. Indeed, it was found that using the recombinant Fab as capture antibody instead of Mab and adding the denatured mouse IgG into the assay buffer were also necessary for these two assays. For consistency, the same assay buffer was used in the tPSA(2C1) assay too, even though the capture antibody was Mab in this assay. The analytical and functional detection limits of the fPSA-N and fPSA(2C1) assays were comparable to the fPSA-I assay's detection limits and the tPSA(2C1) detection limits were also close to the detection limits of the previously published tPSA assay.

In the fPSA-N assay, the unique Mab 4D4 which recognizes PSA without the internal cleavage at Lys145/Lys146 was used as a blocker antibody to inhibit the binding of the tracer Mab 2C1 which has an overlapping epitope. The blocking was stable: 88% of the fPSA-I signal was blocked. The reasons for imperfect blocking may be that the epitopes are not completely mutually exclusive and that the affinity of 4D4 is slightly low due to the rather fast dissociation of the antibody and antigen (**unpublished**, results not shown).

To compensate for the lower affinity 4D4 was used in large excess. Increasing the amount further might improve the blocking efficiency but it was considered impractical as the consumption of the antibody would have been too high.

The concentrations measured with the new assay methods were compared to the concentrations measured from the same patient samples with the previously used methods by Deming regression analysis. The fPSA-N assay gave concentrations that were generally lower than the calculated concentrations. Based on the regression line slope (1.23) and the y-axis intercept (close to 0), the difference between the methods seem to be proportional rather than constant. This is most likely due to differences in assay constructions (for example antibody combinations and assay protocols) which are known to affect assay results as discussed in chapter 2.4.2 (Kort *et al.*, 2006; Stephan *et al.*, 2006b). Furthermore, it is theoretically possible that there are differently cleaved or other kinds of fPSA forms which are not recognized by either fPSA-N or fPSA-I assay but are measured by fPSA assay. Whether this observed difference in measured and calculated concentrations has any clinical impact remains to be seen in the comparative studies to be performed in the future.

In order to obtain fPSA and tPSA concentrations comparable to fPSA-N concentrations new assays with equal protocols and only the minimal necessary changes in the capture or tracer antibodies were developed. The new fPSA(2C1) and older fPSA assays seemed to agree rather well based on the Deming regression analysis as the slope was 1.03 even though the differences in some individual samples were high. The tPSA concentrations measured with the new tPSA(2C1) assay were lower than the concentrations measured with the previously used assay method (slope 0.86) emphasizing a need for the new assay to gain comparable results. As stated, the differences in antibody combinations and assay constructions are the probable reasons for the disagreement between the old and new methods.

The specificity of the fPSA-N assay was as expected because the measured fPSA-N proportions of five seminal plasma pools containing different amounts of intact and nicked PSA were similar to previous results (Nurmikko *et al.*, 2000; Zhang *et al.*, 1995). However, the pools D and E showed somewhat lower proportions of fPSA-N than expected which may be due to the presence of PSA forms unrecognizable to our immunoassay (Mattsson *et al*., 2008).

In the future, the fPSA-N assay should be standardized with pure nicked PSA standard material. The development of a purification method for fPSA-N is ongoing. As the assay method is rather complicated and long, it should be streamlined. To redesign the assay the binding affinity of the 4D4 antibody needs to be improved. One way to achieve this is to develop 4D4 Fab fragment and genetically engineer the binding site of the Fab to exhibit slower off-rate for dissociation from the antigen. This process has been started and results are expected within the next year. Another option is to develop an fPSA-N specific binder for example from a phage display library of Fab fragments.

6.2 Concentrations of fPSA forms and thK2 in blood samples

Because the optimized assays were used to measure the clinically defined radical prostatectomy patient samples for the first time in this study it is of interest to compare these concentrations of fPSA forms and hK2 in blood circulation to the previously reported values. In the different cohorts, the median fPSA-I concentration in non-cancer patients was between 0.36 and 0.49 ng/mL (**III, IV**) and between 0.41 and 0.55 ng/mL in cancer patients (**II–IV**). The previously reported median values for fPSA-I measured with original assays are 0.39–0.43 for non-cancer patients and 0.47–0.49 for cancer patients (Nurmikko *et al.*, 2001; Steuber *et al.*, 2005; Steuber *et al.*, 2002; Steuber *et al.*, 2007a).

The median calculated fPSA-N concentration in non-cancer patients was between 0.35 and 0.54 ng/mL (**III, IV**) while the concentration in cancer patients was 0.24–0.43 ng/mL (**II–IV**). Despite the variation between different sample panels, these results are almost within the same range with previously published results showing median concentration range of 0.41–0.53 ng/mL for non-cancer patients and 0.34–0.50 ng/mL for cancer patients (Nurmikko *et al.*, 2001; Steuber *et al.*, 2005; Steuber *et al.*, 2002; Steuber *et al.*, 2007a).

The proportions of different fPSA forms in the total fPSA fractions are interesting as the combinations of the subforms and fPSA seem to bring new information about the prostate status. In men without cancer 47% of fPSA was fPSA-I and 53% was fPSA-N (calculated concentrations) in the Rotterdam cohort (**III, unpublished)**. The proportions for fPSA-I and fPSA-N in men with a benign disease were 29% and 28%, respectively, when the fPSA-N concentrations were directly measured in another smaller sample set (**IV**). It is interesting that in the latter cohort the proportions of fPSA-I and fPSA-N do not add up to 100%. Whether this is caused by assay differences or the theoretical fPSA forms that our assays for fPSA-I and fPSA-N may not recognize but the assay for tPSA recognizes, remains unclear. The latter is possible because of the variety of the reported PSA isoforms for example in seminal plasma (Mattsson *et al.*, 2008). The Rotterdam cohort proportions are calculated with the older, established fPSA assay and the latter with the new fPSA(2C1) assay but as these two assays showed rather good agreement the differences in fPSA measurement may not be the main reason for fPSA-I and fPSA-N not adding up to 100% of fPSA.

In cancer patients the median proportion of fPSA-I from fPSA was 56–65% (**II–IV, unpublished**) while the proportion of calculated fPSA-N concentration was 40–44% (**II, III**). When the proportion of the directly measured fPSA-N (24% in **IV**), and the median fPSA-I concentration in the same cancer patients are added up the result is 88% which is closer to the 100% of total fPSA than in patients with a benign disease where fPSA-N and fPSA-I comprised 57% of fPSA. The properties of the fPSA-N measurement may be one probable cause for this: the blocking may work proportionally on samples containing different amounts of fPSA-I even though it seems to be stable with different concentrations of standard material. This question should be addressed in the future when the fPSA-N assay is further optimized and validated.

The median concentration of thK2 is 0.075–0.085 ng/mL in the cancer patients of the Rotterdam and Göteborg cohorts (**III**). This is in accordance with previously published median concentrations of cancer patient samples (0.065–0.105 ng/mL) which were measured with the original (or a similar) immunoassay method (Steuber *et al.*, 2007a; Vaisanen *et al.*, 2004; Vaisanen *et al.*, 2006; Vickers *et al.*, 2007). In the men without cancer the median thK2 concentrations were 0.045–0.067 ng/mL. This is in the same range as previously reported values which were between 0.037–0.093 ng/mL for men without cancer (Steuber *et al.*, 2005; Steuber *et al.*, 2007a; Vickers *et al.*, 2007).

6.3 Clinical significance of fPSA and hK2 forms

Diagnosing and treating patients with indolent prostate cancer which is not likely to cause any harm to the patient, in other words over-diagnosis and overtreatment, are problems that cause not only unnecessary anxiety, discomfort and a risk of side-effects for the patient but also significant costs to the healthcare systems (Schroder *et al.*, 2009). One of the main research questions during the last few years has been how to detect the aggressive cases of cancers that need to be treated and leave the indolent cases to be detected only until they later become significant, if they ever do.

Previous studies have compared the fPSA-I concentrations in patients with prostate cancer and benign prostatic hyperplasia with results indicating that fPSA-I seems to be more closely related to cancer than to BPH (Nurmikko *et al.*, 2001; Steuber *et al.*, 2005; Steuber *et al.*, 2002) and that it could aid in the discrimination of these two patient groups.

Of note, the fPSA-I measured with our immunoassay includes also proPSA isoforms (Mikolajczyk *et al.*, 2004) of which especially [-2]proPSA (or p2PSA) has been associated with cancer tissue (Mikolajczyk *et al.*, 2004; Mikolajczyk *et al.*, 2001). The p2PSA has been included in the Beckman-Coulter prostate health index phi (Le *et al.*, 2010) and a recent study by Guazzoni *et al.* suggest that p2PSA and its derivatives, including phi, might help in predicting the prostate cancer pathology (Guazzoni *et al.*, 2012).

In comparison, study **II** was the first one to examine the association of the fPSA-I and fPSA-N concentrations with pathological cancer stage and grade to address their possible usefulness in predicting the cancer aggressiveness. Although the correlations were not strong, a trend showing F/T PSA, N/T PSA and I/T PSA ratios to be lower in patients with more adverse cancer pathology was found to exist. Because tPSA also correlates with the pathological stage and grade it is possible that it has major contribution to the correlation of the different ratios, especially with TNM stage and WHO grade. It is notable that in patients with tPSA < 10 ng/mL fPSA-N, as a single marker, correlated with Gleason score and was significantly lower in patients with high grade cancer than in patients with lower grade cancer, irrespective of how the patients were divided into different groups by the Gleason grade. Thus, it is likely that fPSA-N and, because of the calculation used, fPSA-I have an independent contribution to the correlation of the above mentioned ratios and Gleason grade as well as to their ability to separate different Gleason grade groups. In addition, it is possible that fPSA-N might be used as a marker for less aggressive types of

cancer in this patient group but most likely it would have to be combined with other biomarkers or clinical information to reach clinical significance.

The concentrations of different PSA forms overlapped in different groups based on pathological stage or grade and, therefore, the single markers did not have prediction of cancer pathology in an individual patient. However, as the above mentioned ratios were statistically different between some of the groups the markers might work as a combination in a statistical model. Several different models have been created for both prognostic and predictive use, as discussed in chapter 2.5 (Gosselaar *et al.*, 2008; Kranse *et al.*, 2008; Makarov *et al.*, 2007; Roobol *et al.*, 2012; Schroder and Kattan, 2008; Shariat *et al.*, 2008; Steyerberg *et al.*, 2007).

However, we did not create a model based on the radical prostatectomy patient data because we did not have enough follow-up data of the patients to evaluate the model nor did we have access to other pre-clinical data such as the clinical stage or biopsy grade making the comparison with currently used methods difficult. Furthermore, the number of patients was not quite high enough for creating and validating such a model.

A statistical model was developed to predict biopsy outcome in men with elevated PSA and participating the European Randomized Study of Screening for Prostate Cancer, or ERSPC (Vickers *et al.*, 2010a; Vickers *et al.*, 2008; Vickers *et al.*, 2010b; Vickers *et al.*, 2010c). The biopsy is uncomfortable to the patient, increases the risk of infections and other side-effects (Loeb *et al.*, 2011; Nam *et al.*, 2010) and obviously increases the costs to the healthcare system, therefore, avoiding the unnecessary biopsies would benefit both the patient and the healthcare system. The model was based on the measurements of tPSA, fPSA, fPSA-I and thK2 – also called as the kallikrein panel – from serum samples. The model was shown to potentially reduce the number of unnecessary biopsies by 49–60% in men without recent screening and 30-40% in recently screened men when a cut-off of 20% cancer risk was used as a threshold for biopsy. The few cases of cancer missed were mostly low-grade cancers typically considered to be over-diagnosed.

In study **III**, the aim was to add information about prostate volume to the kallikrein model and study its effect on the model's predictive accuracy in previously unscreened men with indication for biopsy. The combination of prostate volume and serum tPSA concentration has been associated with prostate cancer risk in several studies (Catalona *et al.*, 2000; Epstein *et al.*, 1994; Freedland *et al.*, 2005). Whether any of the kallikreins or their combinations could replace the volume measurement was also of interest. The volume measurement is acceptable to most men but some find it uncomfortable (Aus *et al.*, 1993; Djavan *et al.*, 2001). It requires some expertise (Ko *et al.*, 2011) to be reproducible and a visit to the urologist is also necessary. Money and time would be saved if the volume measurement could be replaced with a blood draw.

Indeed, the kallikrein model (including age, tPSA, fPSA, fPSA-I and thK2) was equally accurate to the clinical model (including age, tPSA, DRE, TRUS volume) in predicting prostate cancer in biopsy. The predictive accuracy was expressed as AUC (see chapter 2.5.1) the values being 0.763 and 0.774 for the clinical model and 0.767 and 0.809 for the

kallikrein panel in the Rotterdam and Göteborg cohorts, respectively. When the TRUS volume was included in the full laboratory model (kallikrein panel and DRE) the increment of AUC was 0.014 or 0.016 (in the Rotterdam and Göteborg cohorts, respectively) which is considered very small even though it was statistically significant in the Rotterdam cohort. Results were essentially similar when high-grade cancer was predicted, further supporting the kallikrein panel's ability to replace prostate volume in this predictive model.

Our hypothesis was that especially fPSA-N might correlate with prostate volume because Steuber *et al.* had previously shown that in a linear regression model fPSA and fPSA-N were the most important predictors for prostate transition zone volume (Steuber *et al.*, 2005). The fPSA-N correlated moderately with prostate volume (Spearman's correlation coefficient 0.55 and 0.41 in the Rotterdam and Göteborg cohorts, respectively) fPSA-N having the best correlation of the single markers in the Rotterdam and second best in the Göterborg cohorts. Our results are supported by a study in which another internally cleaved free PSA isoform BPSA was found to correlate with prostate volume as well as transition zone volume independent of age (Spearman's correlation coefficient of 0.63 and 0.64, respectively) (Canto *et al.*, 2004). In another study, BPSA and the ratio of BPSA to proPSA were also found to help in distinguishing prostate cancer patients from men without cancer in men with F/T PSA < 15 % as well as in men with tPSA within the range of 4–10 ng/mL (Khan *et al.*, 2004).

The presence of cancer is unlikely to effect the correlation based on a preliminary correlation analysis with the Rotterdam cohort (Pearson's correlation coefficient of log-transformed concentrations of the antigens). The correlation coefficients were similar between the men with and without cancer (results not shown). tPSA and thK2 correlation coefficients had the largest differences between the groups so that the correlation coefficients were higher for the non-cancer group (0.367 vs. 164 for tPSA and 0.45 vs. 0.267). In order to study the effect of cancer to the volume correlation of each antigen a regression analysis should be done but, unfortunately, that was not within the timeframe of the current study.

The fPSA-I, as a more cancer related marker (Nurmikko *et al.*, 2001; Steuber *et al.*, 2002), was not expected to correlate strongly with prostate volume. Another recent study by Guazzoni *et al.* suggest that the ratio of p2PSA to fPSA and phi might help in predicting the prostate cancer in the initial biopsy (Guazzoni *et al.*, 2011). Overall, the studies with different free PSA forms support each other by indicating that they probably would help both in estimating the cancer risk before the initial biopsy and in estimating the cancer aggressiveness preoperatively. However, they are most beneficial when used in combination with other markers, usually with tPSA and fPSA or with some clinical data such as age, DRE or prostate volume.

The biology behind the different PSA forms in circulation is not clear but it has been hypothesized that the PSA leaked from the BPH cells is exposed to proteases and thus cleaved in the extracellular matrix before diffusion into the circulation. The cleavages inactivate the PSA and make it unable to form complexes with its inhibitors. On the other

hand the PSA in cancer tissues might diffuse faster into circulation due to the distorted structure of the cancerous tissue and it is less processed. (Balk *et al.*, 2003; Chen *et al.*, 1997; Stenman, 1997.) This would explain the lower proportion of fPSA in cancer patients' circulation as well as the higher proportion of intact fPSA forms. The volume dependency of the PSA concentrations could be explained by the increased number of epithelial cells in BPH tissue and by the observation that PSA secretion of cancerous cells may actually decrease (Schalken, 2004).

hK2 has been shown to be valuable in the kallikrein panel even though its significance may not be as great as that of the combination of fPSA and fPSA-I (Vickers *et al.*, 2010a; Vickers *et al.*, 2008; Vickers *et al.*, 2010c). Because in many studies hK2 has been associated with tumor volume or cancer aggressiveness it may bring additional information regarding the cancer status (Haese *et al.*, 2003a; Haese *et al.*, 2003b; Haese *et al.*, 2005; Steuber *et al.*, 2005).

Roobol *et al.* suggested that the prostate cancer risk calculator (www.prostatecancer-riskcalculator.com, discussed in chapter 2.5.2) which has been developed based on the ERSPC data (Kranse *et al.*, 2008; Steyerberg *et al.*, 2007) should contain information about the prostate volume whether measured by TRUS or estimated during the DRE (Roobol *et al.*, 2012). The authors suggest that the DRE-based estimation could be used to make the use of the risk calculator easier. It might be possible to replace the volume estimation by the measurement of the kallikreins, which would make the use of the calculator even easier because, instead of inviting the patient to visit a urologist, the sample could be sent to a further analysis and the costs of a clinical visit would be replaced by the costs of the immunoassays. It has been estimated that the kallikrein assays would add less than $100 (approximately 75 €) to the cost of testing the tPSA alone (Vickers *et al.*, 2008).

So far it has not been sensible to study the combination of fPSA-I and fPSA-N in a statistical model like Khan *et al.* have done with proPSA and BPSA (Khan *et al.*, 2004) because the use of calculated fPSA-N concentrations. The calculated fPSA-N concentrations are interdependent of the fPSA and fPSA-I concentrations and thus the information of fPSA-N is already included in the model if the two other concentrations are used. Once the direct fPSA-N immunoassay (**IV**) has been optimized for large scale measurements the relevance of the combination of fPSA-I and fPSA-N is obviously one of the most interesting thing to be studied.

Taken together, the growing number of studies are indicating that internally cleaved fPSA forms (fPSA-N, BPSA) are related to the benign volume of the prostate whereas the intact forms (fPSA-I, proPSA) are more likely related to the presence of cancer. It would be of great interest to study the presence of these forms side-by-side in the very same samples (for example blood samples or tissue lysates from the prostates) to better understand the bigger picture of fPSA forms. They may tell the same story or they may offer independent information regarding the biology behind prostate diseases. Combining the stories might help us in tackling the current problems in prostate cancer diagnostics.

7 CONCLUSIONS

As the systematic PSA based screening for prostate cancer seems to result in over-diagnosis and overtreatment (Schroder *et al.*, 2009), the search for new and better markers for identifying the potentially aggressive types of cancer has been intensive.

Despite that some scientists declared already in 2004 that the "PSA era is over" (Stamey *et al.*, 2004) and the fact that PSA is not cancer specific, it still remains the most important prostate cancer marker. The current trend to combine the information of different isoforms of PSA and possibly some of the newer markers has consolidated the position of PSA also as the future marker for one of the most common types of cancer in the Western world.

This study focused on the measurement of intact and nicked PSA isoforms and free and total hK2 in different clinical settings. The main conclusions based on the original publications are:

I The assay interference observed in the fPSA-I and fhK2 immunoassays was significantly reduced by using recombinant Fab or enzymatically produced $F(ab')_2$ fragments instead of intact monoclonal antibodies in the assays. Furthermore, the heat-denatured mouse IgG was found to be the best blocking agent and it was added to the assay buffer to supplement the bovine IgG used as a regular component in the buffer. The assay redesign did not have any negative effects on the analytical performance of the assays.

II The measured fPSA-I and calculated fPSA-N preoperative concentrations as well as their proportion to tPSA were found to be associated with pathological Gleason grade of the prostate cancer in radical prostatectomy patients, especially in the patients with tPSA concentrations below 10 ng/mL. The strength of the association was moderate. The association of the markers with pathological stage or WHO grade may not be independent of tPSA contribution. Further studies with follow-up data on the patients are needed to clarify the relevance of the isoforms for predicting cancer pathology.

III The kallikrein panel including age, tPSA, fPSA, fPSA-I and thK2 could predict the biopsy outcome equally well to the clinical model including age, tPSA, DRE and TRUS volume in unscreened men with indication to biopsy. The kallikrein panel could be considered to replace the volume measurement in prostate cancer risk estimations. Furthermore, the calculated concentration of fPSA-N was found to correlate with the prostate volume as a single marker.

IV An immunoassay for direct measurement of fPSA-N was developed and evaluated. The assay was specific and had an acceptable analytical and functional performance. The slightly complicated assay method needs further optimization and the standardization should be done in the future with fPSA-N instead of fPSA-I which is currently used.

In conclusion, the use of PSA measurement is shifting from single tPSA measurement towards multimarker measurements combining the information from several serum markers with clinical information, most likely in a statistical model. The free PSA isoforms fPSA-I and fPSA-N seem to contain information additional to total PSA and when combined to hK2 it is possible to estimate the prostate cancer risk even more accurately. The future studies should be able to show whether the kallikrein panel could be used in other clinical settings, for example, to aid in estimating the prognosis after treatment for prostate cancer.

8 ACKNOWLEDGEMENTS

This study was carried out at the Department of Biochemistry, Food Chemistry and Biotechnology, University of Turku during 2005–2012. The study was supported by the European Union 6th framework contract LSHC-CT-2004-50311 (P-Mark), the Finnish Academy (project No. 878541) and the Graduate School of *In Vitro* Diagnostics.

I am grateful to my supervisor, Professor Kim Pettersson: Thank you for your trust in me and for the support and guidance whenever I needed it while growing up as a researcher. I also want to thank you for taking me to the P-Mark meetings which were wonderful possibilities to meet esteemed researchers all over Europe. I'm sure these contacts will be valuable in the future.

I also thank Dr. Pauliina Niemelä, my other supervisor: Thank you for all your support. I truly appreciate that you were able to find the time for all the meetings regarding my study and for commenting on my texts despite your busy schedule. You have cheered me up when I had difficulties, and encouraged me to continue.

Thank you, Adjunct Professor Patrik Finne and Adjunct Professor Hannu Koistinen for reviewing the thesis and for your invaluable comments. I also thank Anu Toivonen for not only reviewing the language of this thesis but also taking care of my mental and physical health at your wonderful oriental dance classes during the past 14 years.

I am grateful to all collaborators influencing my thesis: Dr. Ville Väisänen, my mentor since I started my PhD studies, and Dr. Hans Lilja, the esteemed collaborator for many years, thank you for all your supervision, support and inspiring discussions throughout these years. Dr. Martti Nurmi and Dr. Kalle Alanen, thank you for the invaluable clinical input for the studies. The statisticians Tommi Viitanen, Dr. Sigrid Carlsson, Daniel Sjoberg and Dr. Andrew Vickers, thank you for all the statistical analyses that were too difficult for me to perform. Professor Fritz Schröder and Dr. Monique Roobol, thank you for all the co-operation during the past years; it has been an honor to work with you.

I also want to thank Emeritus Professor Timo Lövgren and Professor Tero Soukka for creating and keeping up a unique and inspiring research atmosphere at our department. I am proud to acknowledge my scientific roots. Of course, all my colleagues, researchers and students are part of the success story of the department and I am happy that I have had the privilege to work with you. Thank you all!

Special thanks to Marja-Liisa Knuuti, Kaisa Linderborg, Görel Salomaa, Marika Silvennoinen and Sanna Koivuniemi for taking care of the ever growing paperwork, Mirja Jaala for keeping the lab working and Marja Maula for picking up all the samples, to Sari Lindgren, Veikko Wahlroos, Pirjo Pietilä and Henri Lähteenmäki who have been invaluable in producing and preparing all those antibodies and other reagents needed and to Pirjo Laaksonen for taking samples and answering all the questions regarding MultiCalc, AutoDelfia or labellings (and for the taukojumppa!).

The colleagues in our office, "Maisemakonttori", you were often the first ones to hear about the ups and downs of my life – thank you for listening and sharing those moments. Our discussions have been fruitful and many problems have been solved with your help. Special thanks to Johanna Vuojola and Henna Hautala for your valuable comments regarding my thesis.

Thank you all the past and present members of MPC (Markers of Prostate Cancer) project for sharing the interests and ideas, for the support and fun moments. Special thanks to Riina-Minna Väänänen for all the shared moments at the department, in different hotels and meetings, and also for reviewing many manuscripts and this thesis. Your support has been more important than you probably can imagine. Henna Kekki, Md. Ferdhos Khan Liton and Saeid Alinezhad, it has been a pleasure to know and work with you. I also wish to thank all the students contributing to the study, I appreciate your work. A very warm thank you to everyone in "BPH meeting" group, especially to Maria Rissanen and Lasse Välimaa, who haven't been mentioned yet. Our meetings have been, despite how we call them, a relief to the pain in the brain...

My dear friends Heidi and Tuomo, Riikka and Mika, Katja and Janne, Maria and Kai, Antti and Sirkku, Riikka and Jarkko, Saara and Tomi, Johanna and Tuomas as well as the families, thank you for the support and great shared moments, I hope you know that you all are important to me. Maria deservers special thanks for the support during the last phases of the thesis project. Thanks to all my scouting and geocaching friends for organizing opportunities to get out the lab and reminding me of the life outside BioCity.

The Kaislaniemi family, Aino-Riitta and Ilpo, Samuli and Virva, Mari and Sara, Maammo and all others, thank you for the very warm welcome into your family. Special thanks to Aino-Riitta for the care and maintenance during the writing process.

Valtava kiitos äidille ja isälle, jotka aina jaksavat uskoa, luottaa ja tukea. Kiitos, että olette olemassa, rakastan teitä. Kiitos myös veljille Kaleville, Harrille ja Ismolle perheineen kaikenlaisesta tuesta, avusta ja seurasta vuosien aikana, olette hurjan tärkeitä kaikki!

Aleksi, love of my life, my own fisherman and rock star. I can't express my gratitude for your mental and sometimes even physical support during this process. Your hugs and kisses, funny words and the amazing songs you sing to me all make me love you more and more each day. And it is so important to know that "niin sinäkin minua".

Mari Peltola

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